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The Journal of Clinical Investigation

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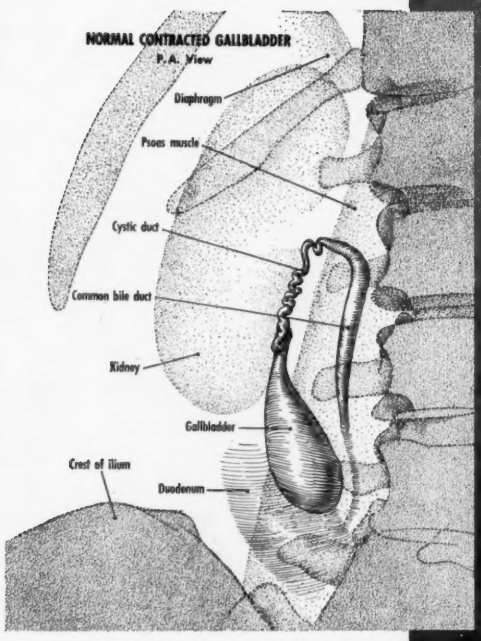

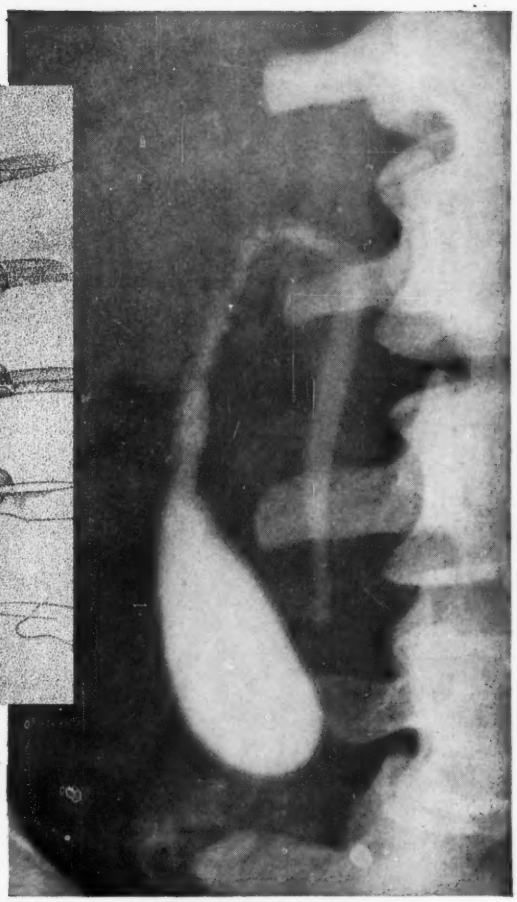

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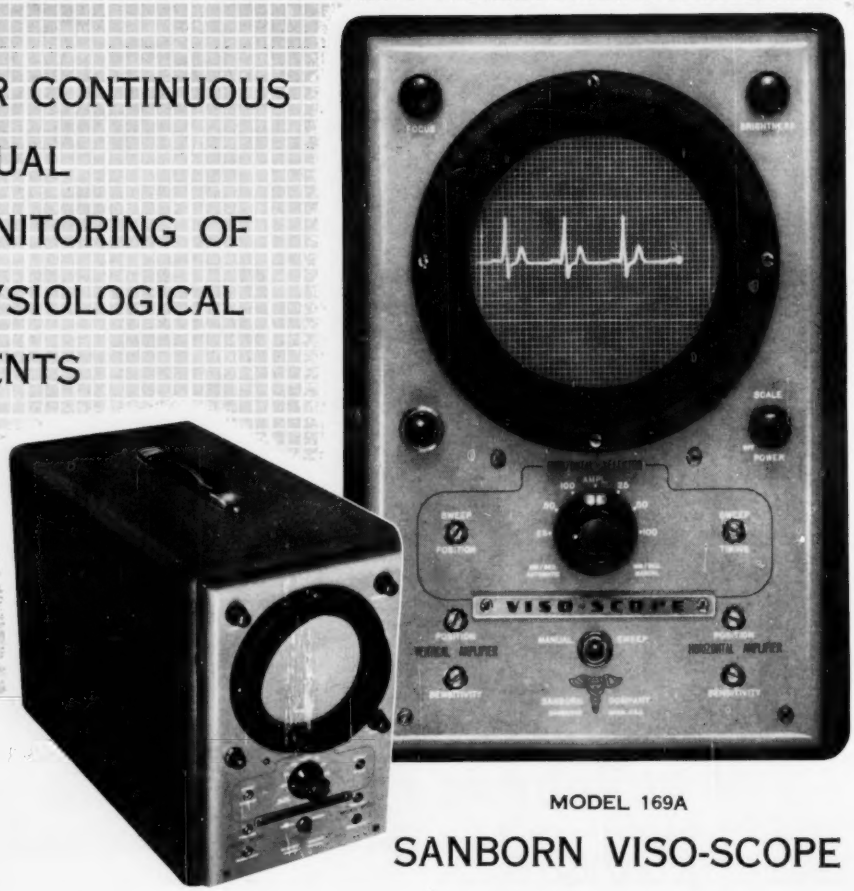

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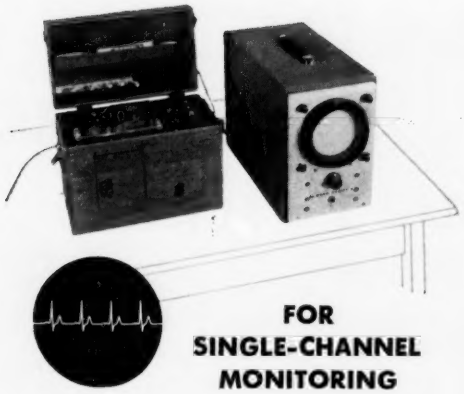

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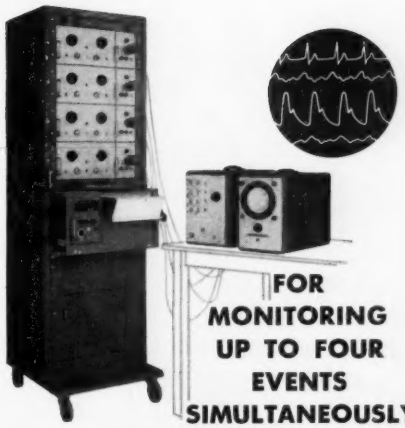

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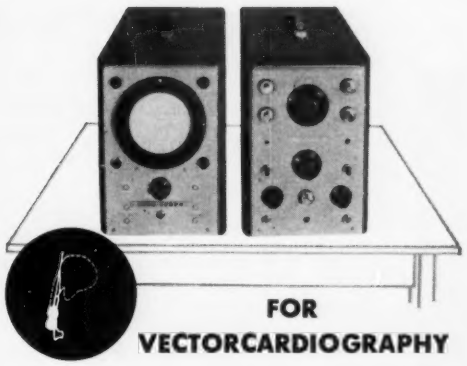

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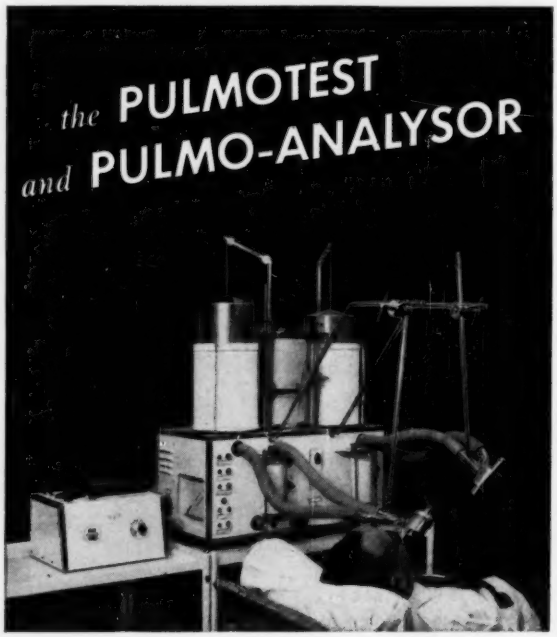

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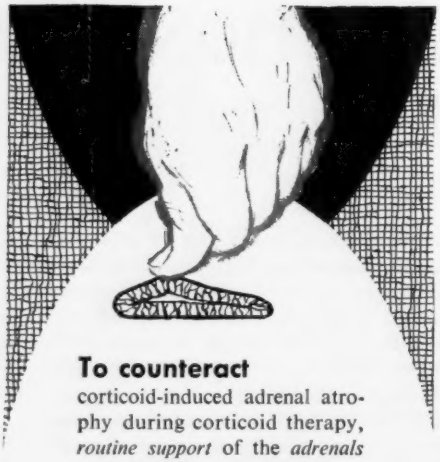

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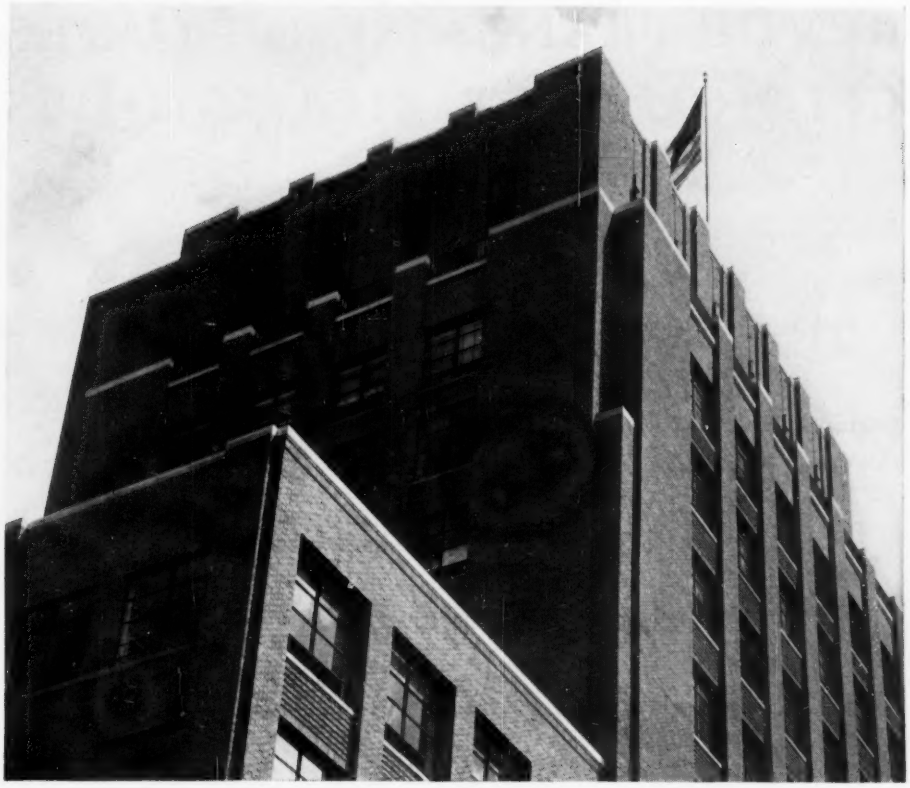

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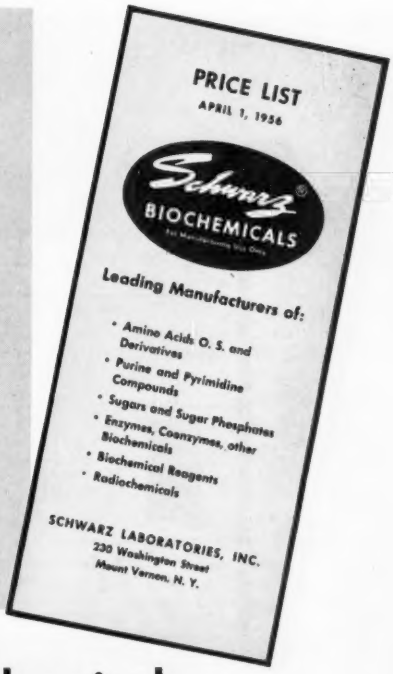

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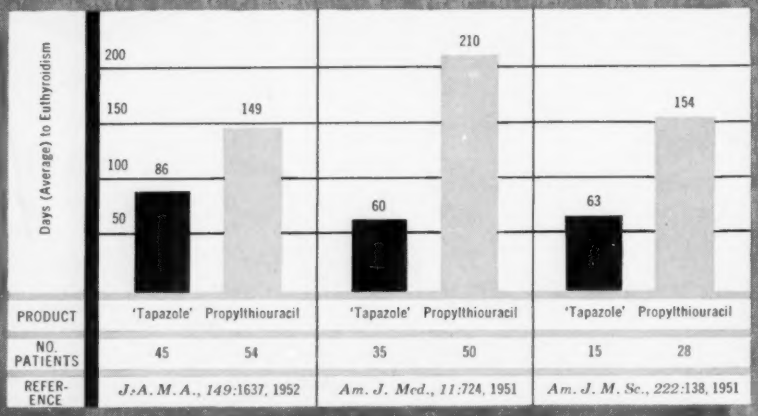

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THE PROPERDIN SYSTEM AND IMMUNITY. IV. THE HEMOLYSIS OF ERYTHROCYTES FROM PATIENTS WITH PAROXYSMAL NOCTURNAL HEMOGLOBINURIA [1]

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Paroxysmal nocturnal hemoglobinuria (PNH) is a rare chronic hemolytic anemia characterized by an acquired defect of the erythrocyte which renders it susceptible to hemolysis by normal human serum. Previous reports have indicated that magnesium and factors resembling the components of complement are required for the hemolysis *in vitro* of PNH erythrocytes by normal serum (1, 2).

Recently a naturally occurring serum protein, properdin, has been described which requires magnesium and complement for its activity *in vitro* (3). Thus, properdin, magnesium, and complement have been termed the properdin system, which participates in the lysis of certain bacteria and the inhibition of certain viruses (4, 5).

The resemblance between the factors required for PNH hemolysis and the constituents of the properdin system prompted this study on the role of the properdin system in the hemolysis of PNH erythrocytes.

METHODS

The test system in all instances was the hemolysis of PNH erythrocytes obtained from three patients with typical PNH whose case histories will be reported elsewhere (6). The red blood cells from all three patients had similar characteristics.

Erythrocytes from oxalated or defibrinated blood were washed three times in isotonic sodium chloride and resuspended to a concentration of 50 per cent in isotonic sodium chloride containing 0.0005 M magnesium chloride. They were stored at 4°C and discarded after 5 days.

Sera: Fresh, clotted venous blood was allowed to remain for 30 minutes at room temperature (20° to 25°C) and then stored overnight at 4°C. Serum was separated from the clot by centrifugation and stored at 4°C. On storage at 4°C serum loses its ability to hemolyze PNH erythrocytes in several days; therefore all sera were used within three days of collection. Serum, however, can be stored at −70°C for at least six months without loss of hemolytic activity. Serum was diluted with isotonic sodium chloride containing 0.0005 M magnesium chloride.

Heated serum was prepared by heating in a 56°C water bath for 30 minutes. Properdin is inactivated in such serum (3).

Hemolytic system: Except where indicated all tests were conducted in a buffered system at pH 6.8 to 6.9. Since minor variations in pH have been noted to result in marked changes in degree of hemolysis, a well buffered system is essential. The buffer was made by dissolving 3.4 gm. of imidazole in 90 ml. of 0.2 M hydrochloric acid, and adding distilled water to 100 ml. This was titrated to pH 6.7 with approximately 0.2 volumes of normal hydrochloric acid (7). All sera were acidified and buffered after heating or other pre-treatment. The addition of 0.1 volume of 0.145 M hydrochloric acid and 0.1 volume of the buffer to one volume of serum, followed by 0.1 volume of the 50 per cent erythrocyte suspension resulted in a stable final pH of 6.8 to 6.9. The mixture was incubated for 15 minutes at 37°C in a water bath. After incubation and centrifugation, hemolysis was scored by inspection on a scale of 0 to +++, or the free hemoglobin was determined quantitatively on an Evelyn photoelectric colorimeter to obtain per cent hemolysis. Changes in the number of transfused cells present in patient's cell population caused the variations in hemolysis observed in different experiments.

Special serum preparations: Serum samples from which components of complement, C'1, C'2, C'3, or C'4 had been removed, referred to respectively as R1, R2, R3, and R4, were prepared as previously described (8).

Properdin was removed from serum by exposing it to zymosan at 17° C (3). Serum rendered deficient of properdin by this means is called RP. It contains all measurable factors of the clotting system and all components of complement in amounts comparable to untreated serum. The RP used in these studies was required to meet the criteria previously established (3, 9).

Purified properdin was prepared by a method described elsewhere (3). It was concentrated to contain 100 to 700 units per ml. in phosphate or barbital buffer and was diluted with isotonic sodium chloride immediately prior

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[2] U. S. Public Health Service Postdoctoral Research Fellow.

to use. Such preparations retained potency for at least 6 months at −20°C.

Resin-treated serum was prepared by the addition of an equal volume of Amberlite IRC-50 (sodium cycle) to serum for ten minutes at room temperature. In this way 99.9 per cent of calcium and magnesium ions were removed (10). Such serum was used immediately after preparation because of the instability of C'1 in the absence of calcium.

Thrombin: Lyophilized thrombin (Parke-Davis and Co.) was dissolved to a concentration of 1000 international units per ml., in isotonic sodium chloride containing 0.0005 M magnesium chloride. The solution was stored at −20°C and thawed immediately prior to use. Fifty units (0.05 ml.) was added to 1 ml. of serum in the PNH test system (11).

RESULTS

1. *Effect of removing properdin on PNH hemolysis*

The removal of properdin from serum rendered that serum inactive for the hemolysis of PNH erythrocytes (Table I). Fresh normal serum was found to be more hemolytic at pH 6.8 than 7.4. At the optimum pH of 6.8, serum from which properdin had been removed, (RP) was completely inactive for hemolysis of PNH cells, even though it contained normal amounts of all the components of complement. Other serum samples from which one component of complement had been removed, R1, R2, R3, and R4, were also inactive for PNH hemolysis, although R2 and R4 contained normal amounts of properdin.

However, as indicated in Table II, the removal of properdin from serum did not inactivate systems dependent on the action of a specific hemolysin, such as paroxysmal cold hemoglobinuria, anti-A or anti-B isohemolysin, amboceptor sheep-cell hemolysin, or anti-human immune erythrocyte hemolysin. The hemolysis of PNH erythrocytes in a serum did not result in a decrease in the level of complement or properdin in that serum. However, the assay methods would not detect small changes in either complement or properdin.

TABLE I

The effect on hemolysis of PNH erythrocytes of removing properdin from serum

Serum	pH	Properdin	Complement	Hemolysis of PNH erythrocytes
Normal	7.4	Normal	Normal	+
Normal	6.8	Normal	Normal	+++
RP	6.8	0	Normal	0
R1	6.8	Normal	Lacks C'1	0
R2	6.8	Normal	Lacks C'2	0
R3	6.8	0	Lacks C'3	0
R4	6.8	Normal	Lacks C'4	0

TABLE II

The effects on PNH and immune hemolytic systems of removing properdin from serum

Type of hemolysis	Hemolysis in whole serum	Hemolysis in RP serum
PNH	Present	Absent
Paroxysmal cold hemoglobinuria	Present	Present
Anti-A, Anti-B	Present	Present
Sensitized sheep cell	Present	Present
Anti-human red cell	Present	Present
Hemolysin from patient with acquired hemolytic anemia	Present	Present

2. *Restoration of the ability to hemolyze PNH cells by the addition of purified properdin to RP serum*

The addition of purified properdin to RP to a final concentration of 5 units per ml. caused marked PNH hemolysis, whereas RP or properdin alone caused no hemolysis (Table III). Properdin prepared from cow or hog serum was also effective. Properdin did not restore hemolytic activity to R1, R2, R3, R4 and heated serum.

Comments: Removal of properdin from serum renders the serum inactive for hemolysis of PNH cells. No other measurable substance is removed, for such sera are essentially unchanged as regards complement and the clotting factors. The substance removed by zymosan behaves functionally as a single entity that is fully capable of restoring hemolytic power to RP. The failure of properdin to restore sera inactivated by other methods again

TABLE III

Restoration of ability to hemolyze PNH cells by the addition of properdin to RP serum

Serum	Added properdin	Hemolysis of PNH erythrocytes
Normal	0	+++
RP	0	0
RP	5 units human	+++
RP	5 units hog	+++
RP	5 units cow	+++
R1	5 units human	0
R2	5 units human	0
R3	5 units human	0
R4	5 units human	0
Heated 56°C	5 units human	0
Isotonic sodium chloride	5 units human	0

demonstrates that other factors similar to or identical with the components of complement are essential for hemolysis of PNH cells. Although the inactivated sera were prepared by accepted methods, one cannot exclude the possibility that there are removed other unidentified substances unrelated to complement, but required for PNH hemolysis. The ability of properdin obtained from several animal species to restore the human PNH system strengthens the initial hypothesis that this factor is a common and basic constituent of serum.

3. *The requirement for magnesium in PNH hemolysis*

Removal of magnesium and calcium from serum by cationic exchange resin rendered that serum inactive for hemolysis of PNH erythrocytes. Addition to resin-treated serum of calcium to a final concentration of 0.0015 M did not restore the hemolytic property. Addition of magnesium in a concentration of 0.0005 M resulted in complete restoration of the hemolytic system. Excesses of magnesium resulted in inhibition of hemolysis, probably because of the significant increase in total ionic strength. Addition of 0.0015 M calcium to 0.0005 M magnesium did not result in an increase in hemolysis greater than that resulting from addition of magnesium alone.

Thus, as previously demonstrated (1), there is an absolute requirement for magnesium in the hemolysis of PNH erythrocytes.

4. *Relation of serum properdin level to the PNH hemolytic activity of serum*

The properdin content of human sera correlated with the hemolytic activity of those sera against PNH erythrocytes. In a typical experiment (Table IV) a normal serum No. 1 containing 4 to 8 units of properdin per ml. caused hemolysis of 34 per cent of the test cells, while another normal serum No. 2 containing 1 to 2 units of properdin per ml. caused only 14 per cent hemolysis. RP alone failed to cause hemolysis, but the addition of 3 units of properdin per ml. gave 23 per cent and 5 units caused 36 per cent hemolysis. There was a relationship between properdin content and hemolytic activity in many untreated sera and on adding properdin to RP. However, some untreated sera failed to show good correlation between properdin level and PNH activity. This inconsistency, presumably due to other limiting variables such as complement components, precludes the use of PNH hemolysis as a means of routine measurement of properdin in serum at this time.

TABLE IV

The relation of serum properdin level to hemolysis of PNH erythrocytes

Serum	Properdin content	Hemolysis of PNH erythrocytes
	units per ml.	*per cent*
Normal—No. 1	4–8	33
Normal—No. 2	1–2	14
RP	0	0
RP + 3 units properdin	3	23
RP + 5 units properdin	5	36

5. *Relation of properdin to the thrombin effect on PNH hemolysis*

In normal serum with moderate hemolytic activity for PNH cells, addition of thrombin has been noted to result in increased hemolysis. This has been the basis of a diagnostic test for PNH (11), and forms an important part of a theory proposed by Crosby relating PNH hemolysis to the clotting system. Accordingly, the possible relationship between thrombin and properdin effect was investigated.

Both commercial and "pure" thrombin (Parke-Davis and Co.) were found to contain properdin activity, varying from one unit to four units of properdin in every 50 units of thrombin, which is the amount used in the thrombin test. Neither properdin nor thrombin was hemolytic alone, but the addition of equivalent amounts of thrombin or properdin to serum resulted in equal augmentation of hemolysis of PNH cells (Table V). Thus, a serum initially containing 2 units per ml. of properdin caused 21 per cent hemolysis. Addition of 50 units of thrombin "A", a preparation containing 2 units of properdin in each 50 units of thrombin, caused 45 per cent hemolysis; addition of 2 units of properdin resulted in 48 per cent hemolysis. On another occasion, using thrombin "B", a preparation containing approximately 4 units of properdin in each 50 units of thrombin, addition of 50 units of thrombin caused increase in hemolysis in serum from 11 per cent to 54 per cent. Five units of properdin gave an increase in hemolysis from 11 per cent to 56 per cent.

TABLE V

Effects on hemolysis of adding properdin and thrombin to serum

Date	Thrombin	Serum mixture	Hemolysis of PNH erythrocytes
			per cent
9-25-54	Thrombin "A"		
	(50 units contains 2 units of properdin)	Serum	21
		Serum + 50 units thrombin	45
		Serum + 2 units properdin	48
1-14-55	Thrombin "B"		
	(50 units contains 4 units of properdin)	Serum	11
		Serum + 50 units thrombin	54
		Serum + 5 units properdin	56
		RP	0
		RP + 50 units thrombin	27
		RP + 5 units properdin	24
		R3 + 50 units thrombin	0
		R3 + 5 units thrombin	0

The addition of 50 units of thrombin "B" or 5 units of properdin to RP resulted in 27 per cent hemolysis, but neither caused hemolysis when added to R3 or heated serum.

Larger amounts of thrombin have an anticomplementary effect on serum and could not be tested.

Comments: The detection of properdin in thrombin preparations and the evidence that 1) the hemolytic property of thrombin corresponds to its properdin content, and 2) thrombin specifically restores an RP serum to hemolytic activity, suggest that properdin is the active principle responsible for the effect of thrombin. Accordingly, the activity of thrombin may be due to the addition of properdin contained in thrombin rather than to the inactivation of a proposed inhibitor by thrombin *per se*. The coexistence of properdin with thrombin in preparations of the latter is not surprising, for both are β-euglobulins with similar iso-electric points. It was not possible to remove properdin from thrombin preparations with zymosan because of the requirement for magnesium and the components of complement.

DISCUSSION

Both complement and the clotting system have been implicated in the hemolysis *in vitro* of PNH erythrocytes. Originally Ham and Dingle (2) showed that destruction of any component of complement resulted in loss of hemolytic activity of serum, and that addition of guinea pig complement to normal serum caused increased hemolysis. However, serum complement did not appear to be depleted during the hemolysis of PNH cells, and subsequent studies indicated that the hemolytic activity of a serum could be destroyed without loss of conventional complement (12). Thus, complement alone did not appear to be responsible for hemolysis of PNH cells, although factors similar to complement appeared to be necessary for the hemolytic reaction.

Crosby and Dameshek (13) proposed that the PNH hemolytic factor was related to ac-globulin, but Harris, Jordan, Pillemer, and Desforges (1) clearly showed that ac-globulin was not essential for hemolysis of PNH cells. More recently, Crosby (14) has proposed a complex of four accelerators and inhibitors of the PNH system related to the clotting mechanism. This theory had its origin in the observed clinical tendency to thrombosis in patients with PNH, and it is based in large part on the action of thrombin in causing augmentation of PNH hemolysis *in vitro*. None of the four proposed factors has been identified in support of this theory.

The current study offers evidence that the properdin system, a natural defense mechanism of human serum, is required for PNH hemolysis. In every manner tested the PNH system is comparable in requirements and behavior to the reactions of the properdin system with zymosan, bacteria and viruses. All these reactions require magnesium and factors indistinguishable from complement along with properdin, and all are dependent on a specific temperature and pH.

These observations support the findings of Ham and Dingle (2) that factors resembling complement are necessary for the hemolytic reaction against PNH erythrocytes. The demonstration of the participation of properdin in PNH hemolysis and its relation to complement may explain the hitherto confusing observations that 1) there is no utilization of complement during hemolysis, and 2) hemolytic activity may disappear without depletion of total complement. That there are additional undescribed factors affecting hemolysis is entirely possible.

These studies may identify one factor of the complex proposed by Crosby, for it is probable that properdin is one of the heat labile factors of earlier authors and may be the heat labile acceler-

ator of Crosby. Further, properdin activity is probably responsible for the effect observed when thrombin is added to serum. The existence of a heat stable inhibitor which is inactivated by thrombin was proposed on the evidence that augmented hemolysis resulted from addition of thrombin to serum and its subsequent removal by barium sulfate. Since properdin is present in thrombin preparations and is not removed by barium sulfate, the "thrombin effect" could well be due to the addition of properdin, a procedure which has been shown to augment PNH hemolysis. Properdin has no demonstrable relationship to the clotting system, as evidenced by the fact that properdin preparations contain no activity similar to the described clotting factors, and a serum or plasma from which properdin has been removed is normal for known clotting factors when compared to an untreated control (3).

Thus, these studies indicate that the properdin system, consisting of properdin, components indistinguishable from complement, and magnesium, is required for the hemolysis of PNH erythrocytes *in vitro*. The earlier observations on the role of the properdin system in bacterial destruction and virus neutralization (4, 5) and the requirement of properdin for hemolysis *in vitro* of PNH cells, suggest that hemolysis of PNH erythrocytes may be due to the interaction of properdin with the stroma of the intact erythrocytes. It has already been suggested that the final destruction of PNH cells is due to a proteolytic enzyme system (1), and the relation of properdin to such a system awaits further investigation.

SUMMARY

1. Similarities between the factors required for PNH hemolysis and the factors comprising the properdin system are pointed out.

2. Removal of properdin from serum renders the serum inactive for PNH hemolysis but not for immune hemolytic systems; readdition of properdin derived from several species restores PNH hemolytic activity to such serum.

3. A relationship exists between the PNH hemolytic activities and properdin titers of sera. The addition of properdin to weakly lytic sera also augments hemolysis.

4. The ability of thrombin to affect PNH hemolysis may be due to the presence of properdin in samples of thrombin.

5. The PNH hemolytic system of normal serum includes properdin, factors resembling the components of complement, and magnesium.

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THE PRODUCTION AND IDENTIFICATION OF ANTIBODIES TO INSULIN AND THEIR USE IN ASSAYING INSULIN [1]

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A specific method for the detection of small quantities of insulin in body tissues and fluids might help to resolve some of the problems connected with the mechanism of action and hormonal interrelationships of insulin. The biological assay methods for small quantities of insulin (2–4) are based upon the effect of insulin on glucose metabolism. Although very sensitive, these methods have several disadvantages. They are technically difficult, are dependent upon the physiological effect of insulin, therefore, subject to considerable biological variation, and consequently may not be specific for insulin *per se*. A method specific for insulin and independent of biological variation would, therefore, be highly desirable.

Since serological methods are highly specific and independent of biological variation, an immunological approach to the measurement of insulin in tissues and body fluids appeared promising. Moreover, it has been noted here and in other laboratories (5–8) that the hemagglutination technique employing tannic acid and protein treated red cells is highly specific and also very sensitive. It was suggested (8), however, that greater precision in the measurement of antigen could be attained by converting the hemagglutination to a hemolytic reaction with the addition of complement. In this way the reaction could be measured colorimetrically. This conversion was impossible with tannic acid-treated cells since they often are lyzed in the presence of complement alone (7, 9). Consequently other methods of coupling protein to red cells were sought. The method using bis-diazotized benzidine, as described by Pressman, Campbell, and Pauling (10), has been modified and was used in the present study.

The antigenicity of insulin has been indicated by several investigators (11–15). Barral and Roux (11) and later Bernstein, Kirsner, and Turner (12) elicited anaphylactic responses to insulin in guinea pigs. Lewis (13) observed the Schultz-Dale reaction in uteri of guinea pigs sensitized with insulin. By the use of the complement fixation, Wasserman, Broh-Kahn, and Mirsky (14) were able to demonstrate antibodies to insulin in six of eleven immunized rabbits. Lowell and Franklin (15) induced resistance to the hypoglycemic action of insulin in four of twenty-four rabbits by repeated injections of insulin.

Evidence is presented here that antibodies to insulin can be produced consistently in rabbits. These antibodies, as well as antibodies in patients resistant to insulin, can be detected by hemagglutination and hemolysis of insulin-conjugated erythrocytes. Methods are also presented for the assay of microgram quantities of insulin in pure solutions. Evidence that rabbit anti-insulin serum is specific for insulin, and that there is a high degree of serological cross-reactivity among insulins from various species is presented in the next paper (16).

MATERIALS [2] AND METHODS

The preparation of bis-diazo-benzidine. Bis-diazobenzidine (BDB) was prepared according to the method of Kabat and Mayer (17). It was essential to maintain the temperature of the reaction mixture below 0° C. The final product was a pale yellow solution. It could be stored for an indefinite period of time if frozen at − 80° C and maintained at − 35° C to − 40° C.

Normal rabbit serum. Normal rabbit serum (NRS) was prepared by heating serum from non-immunized rabbits at 56° C for 30 minutes and absorbed with an equal volume of washed, packed sheep erythrocytes for 15 minutes at room temperature. Normal rabbit serum diluted 100 times (1/100 NRS) was used as the diluent in the hemagglutination titrations.

Erythrocyte preparations. [2] Either sheep or rabbit blood diluted with Alsever's solution (18) was used as the source of erythrocytes. The blood was not used for

[1] A preliminary report of this work was presented before the American Association of Immunologists at Chicago in the Spring of 1953 (1). This study was supported in part by a grant from Eli Lilly and Company.

[2] The authors wish to thank the Eli Lilly Co. for the various preparations of insulin.

more than one week and was kept at 5° C while not in use.

Complement preparations. Dried or fresh frozen guinea pig serum was used as the source of complement. All dilutions of complement were made with 0.9 per cent saline. Fresh guinea pig serum was separated from clotted blood within four to five hours after bleeding. The clot was allowed to shrink at room temperature. The serum was quick frozen at − 80° C in an alcohol-dry ice bath and stored at − 35° to − 40° C. Fresh frozen guinea pig serum treated in this manner, retained full hemolytic activity for at least six months.

Isotonic phosphate buffer. One part of 0.219 M Na_2HPO_4 was added to four parts of 0.219 M NaH_2PO_4 to make up a stock solution of ionic strength of 0.3 (twice isotonicity) at pH between 7.35 to 7.4. This stock phosphate buffer was diluted with an equal volume of H_2O to prepare isotonic phosphate buffer.

Insulin preparations. The insulin used in these experiments was derived from either of two lots: Lot No. 535664 or Lot No. 466368. Lot No. 535664 consisted of a preparation of zinc crystalline beef insulin which assayed 27 units per milligram. Lot No. 466368 represented a special trypsin-treated insulin prepared from beef. It probably contained small amounts of trypsin and possibly some other proteins; however, it was free of glucagon (hyperglycemic factor) activity.

The insulin was dissolved in a small volume of 0.005 N NaOH and then diluted to the desired volume with isotonic phosphate buffer, pH 7.35 to 7.4. All stock solutions contained one milligram per milliliter. The solutions were stored at 5° C when not in use. No insulin solutions were used after three days from the time they were prepared.

Immunization. The antigen was prepared by suspending 50 mg. of crystalline zinc insulin in 10 ml. of saline to which was added 0.5 ml. of 1 per cent alum ($KAlSO_4$). Enough 1 N NaOH was then added to the preparation to make it just faintly acid to hydronium ion paper.

Adult white rabbits of either sex weighing between 3 and 4 kilograms were injected in the marginal ear vein with alum-precipitated insulin. In order to prevent hypoglycemia, 25 ml. of 40 per cent glucose was given subcutaneously in the nape of the neck immediately following the insulin injection.

All of the collected sera were inactivated at 56° C for 30 minutes and then absorbed with an equal volume of washed, packed sheep erythrocytes. These rabbit antisera were stored at − 35° C until they were tested.

Preparation of insulin conjugated erythrocytes. A mixture of saline and insulin solution (1 mg. in 1 ml.) was prepared by adding 1.2 ml. of saline to 2.4 ml. of insulin solution. To this mixture, 0.05 ml. of washed packed sheep or rabbit erythrocytes were added. One-half milliliter of BDB, diluted 1/15 with isotonic phosphate buffer pH 7.4, was then added to this cell suspension. With the addition of the phosphate buffer the BDB turned from a pale yellow to a deep reddish-brown. The suspension of cells, saline, insulin, and BDB was then gently mixed, allowed to stand at room temperature for ten minutes, and centrifuged at 1700 rpm for five minutes. The supernatant was decanted, and the cells were suspended in 3.5 ml. of 1/100 NRS. The insulin sensitized cells were then re-centrifuged at 1700 rpm, the supernatant again decanted, and the cells resuspended in 2.5 ml. of 1/100 NRS.

There was no hemoglobin in these supernatants. Microscopically the cells appeared as bi-concave discs; no ghosts or evidence of agglutination could be observed. The insulin sensitized BDB erythrocytes could be used for hemagglutination and hemolytic reactions for a period of at least five hours after preparation.

It was necessary to determine the optimal ratio of BDB to insulin for the sensitization of the erythrocytes. When excess insulin was used, the cells were not agglutinated by the insulin antisera. If too little insulin was used, agglutination and lysis occurred after the addition of BDB to the cell suspension. The ratio of BDB to protein used in sensitizing the red cells was dependent upon the particular protein involved (19). There was little variation of this ratio, however, when different batches of BDB were used with the same protein.

Preservatives, such as phenols, and amino acids such as glycine, when present in the reaction mixture may interfere with the conjugation of protein to the red cell surface (19). It is believed that such compounds react with BDB and thereby prevent the conjugation of protein to the erythrocyte surface by BDB (19).

The detection of insulin antibodies by hemagglutination. Two series of doubling dilutions of each test antiserum ranging from 1/10 to 1/5120 were prepared in 10 × 75 mm. test tubes, with 1/100 NRS as the diluent in a final volume of 0.5 ml. To the experimental series 10 μg. of insulin in 0.1 ml. was added to each test tube and to the controls 0.1 ml. of saline was added to each test tube. After mixing, 0.05 ml. of insulin conjugated erythrocytes was added to all the test tubes, thus making a final volume of 0.65 ml. Controls containing 0.05 ml. of a 2 per cent suspension of washed, unsensitized erythrocytes and 0.6 ml. of a 1/10 dilution of antiserum were run in duplicate at all times. All tubes were shaken in order to disperse the cells evenly and allowed to stand for 3 to 4 hours. Precautions were taken not to disturb the cells while they were settling.

Agglutination was determined by the pattern of sensitized erythrocytes at the bottom of the tube. In the absence of agglutination, a discrete button of red cells was observed. Slight agglutination resulted in the formation of a halo surrounding a central film. Positive agglutination resulted in an evenly distributed sheet of erythrocytes. When agglutination was maximal, this sheet was wrinkled and folded. It is important that the test tubes used (10 × 75 mm.) be uniform and that they do not have distorted bottoms. A detailed description and photograph of these reactions has been presented elsewhere (7). Arbitrary criteria for the presence of antibodies were established as follows:

1. The agglutination of insulin-sensitized erythrocytes by the test antiserum.

2. Agglutination was inhibited when the test antiserum was preincubated with crystalline insulin.

3. The absence of agglutination of non-sensitized erythrocytes by the test antiserum.

The *titre* of any given insulin antiserum was designated as the *highest* dilution in which there was definite evidence that agglutination had taken place.

The hemolytic titration of insulin antiserum. As in the agglutination titration, serial dilutions of antiserum were prepared in duplicate. To one series was added insulin (10 μg. in 0.1 ml.) and to the second, saline. These serial dilutions were cooled to 0° C in an ice bath, and 0.5 ml. of 1/10 guinea pig serum, diluted with saline, was added, thus increasing the volume to 1.1 ml. To each tube 0.15 ml. of insulin-conjugated erythrocytes was then added, bringing the final volume to 1.25 ml. The cells were then evenly dispersed by shaking, incubated at 37° C for 30 minutes, centrifuged at 1700 rpm for 10 minutes and the supernatant decanted and saved. The relative amounts of hemoglobin released into the supernatants were measured quantitatively with the Beckman spectrophotometer at a wave length of 576 mμ. The Lowry and Bessey microadaptation (20) of the Beckman spectrophotometer was used. All of the samples were read against water. The magnitude of the blank from non-specific hemolysis varied with different batches of complement. No corrections for this non-specific hemolysis were made in this study. However, it was subsequently learned that the non-specific hemolysis could be reduced considerably if the guinea pig serum was previously absorbed with sheep erythrocytes, as described in the following paper (16).

TABLE I

The appearance of antibodies to insulin in adult white rabbits

Rabbit No.	No. of injections of alum-precipitated insulin	Volume of each injection ml.*	No. of days before appearance of antibodies to insulin		First observed titre of antiserum
			After first injection	After last injection	
R-1	5	0.2	19	9	1 80
R-2	5	0.2	19	9	0
R-3	5	0.2	19	9	0
R-9	7	0.3	14	2	0
R-10	9	0.3	61	8 19†	0
R-13	3	0.5	8	2	0
R-14	6	0.5	16	3	1 320
R-15	5	0.5	11	3	1 40
R-18	10	0.5	130	2	1 640
4 N 3	9	0.5	131	2	1 160
4 M 0	8	0.5	131	3	1 20
A-1	6	0.5	126	96	1 40
A-2	7	0.5	66	10	1 20
A-3	7	0.5	64	8	1 640
A-4	7	0.5	66	10	1 160
A-5	7	0.5	202	7	1 1280
A-6	9	0.5	202	7	1 160

* Each ml. of alum-precipitated insulin contained 5 mg. of insulin. Prior to immunization the anti-insulin titre in all rabbits was zero.

† R-10 was tested both 8 and 19 days after the last injection—on both of these occasions the serum titre was 0.

When the concentration of hemoglobin was plotted against the antiserum dilutions, two curves were obtained (Figure 2); one corresponding to the dilutions of antiserum with which saline was preincubated, and the other to those where 10 μg. of insulin was preincubated. Consequently it was possible to estimate the degree of inhibition of lysis of insulin-sensitized erythrocytes at any given dilution of antiserum. This information was essential in determining the dilution of antiserum required in running a standard curve for assaying insulin as described in a later section on the insulin assay.

Ring and precipitin reactions. The ring reaction was performed as described by Boyd (21). Various concentrations of insulin in buffer were carefully laid over the antiserum in a capillary tube. The tubes were kept at room temperature for 12 to 24 hours, and observed for the appearance of an interphasic precipitate.

The precipitin reaction was carried out in 10 × 100 mm. glass tubes. One-tenth milliliter of varying concentrations of insulin was mixed with 0.1 ml. antiserum and 0.1 ml. of saline. The tubes were then incubated at 37°C for 1 and 1/2 hours and placed in the refrigerator overnight, and observed for the appearance of turbidity or precipitate.

RESULTS

Characteristics of rabbit antibodies to insulin

The production of insulin antibodies required a minimal dose of antigen (2.5 mg. of alum-precipitated insulin in 0.5 ml.) given 6 to 10 times for a period varying from 16 to 202 days. In Table I, the first five animals received a dose of less than 0.5 ml. (2.5 mg.) of alum-precipitated insulin per injection. Only one of these animals produced antibodies. The remaining twelve animals received 0.5 ml. (2.5 mg.) of an alum-precipitated insulin per injection. Eleven of these animals developed antibodies to insulin. The one animal which did not, died following the third injection. In the eleven animals which produced antibodies to insulin, a minimum of 5 and a maximum of 10 injections were required.

In the majority of animals the antibodies to insulin appeared approximately 2 to 6 months after the first injection of antigen. It is possible that antibodies not detectable by the methods employed may have been produced sooner.

Since a large amount of glucose was administered following each injection of antigen, the injections were spread over a period of two to three months. In this way, the rabbits were exposed to less trauma per unit time, and the risk of infection was decreased.

A specific anamnestic response was observed in all rabbits which had previously developed antibodies. In two animals a single injection of alum-precipitated insulin was followed by a rise of the anti-insulin titre to a peak in seven to nine days (Figure 1). The antibody titre then decreased to 1/10 within 4 months. Alum-precipitated insulin injected at subsequent intervals of two months elicited progressively higher anti-insulin titres. This occurred despite the fact that the anti-insulin titre had returned to very low levels before each of the subsequent injections (Figure 1).

It appears, therefore, that repeated intravenous injections of adequate amounts of alum-precipitated insulin over a sufficiently long period of time can consistently elicit antibodies to insulin in the rabbit.

It was possible to separate the antibodies from the remaining serum proteins by the method of Nicol and Deutsch (22), in which the antibody-rich gamma globulin fraction plus some traces of beta globulin are precipitated from a diluted serum. Seventy per cent of the antibody activity was recovered in the gamma globulin fraction which represented about 10 per cent of the total serum proteins.

Ring and precipitin reactions

Rabbit antiserum (4 MO.) which had a titre of 1/80,000, and other lowered titred sera were studied. The concentration of crystalline beef insulin employed ranged from 38 to 300 micrograms per milliliter. It was realized that even 38 micrograms might constitute excess antigen for these antisera

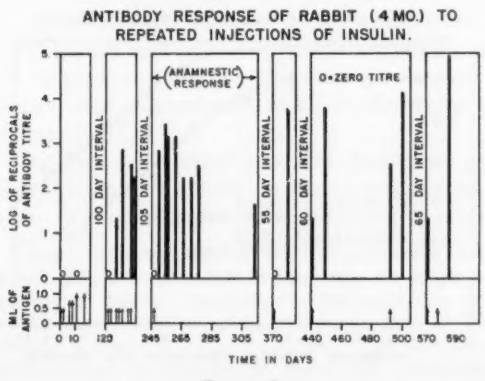

FIGURE 1

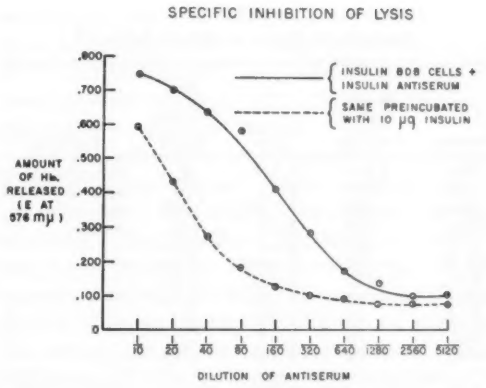
SPECIFIC INHIBITION OF LYSIS
INSULIN 808 CELLS + INSULIN ANTISERUM
SAME PREINCUBATED WITH 10 μg INSULIN
AMOUNT OF Hb. RELEASED (E AT 576 mμ)
DILUTION OF ANTISERUM

FIGURE 2

and soluble, undetectable antigen-antibody complexes might result. However, insufficient antiserum was available for a more systematic study of the precipitability of these antisera with smaller amounts of antigen. All reactions were negative.

Attempts to produce skin sensitivity to insulin

Franklin and Lowell (23) produced skin sensitivity to insulin in intensively immunized rabbits. A number of rabbits possessing high hemagglutination titres (1/640 to 1/80,000) were injected intradermally with 20 micrograms of insulin. Other normal rabbits were injected in several sites with 0.1 ml. of antiserum 4 MO. (titre 1/80,000). Twenty-four hours later, these same areas were injected with insulin (20 to 100 micrograms). As controls in both actively and passively immunized rabbits, equivalent concentrations of bovine gamma globulin were injected into other skin sites previously infiltrated with the anti-insulin serum. All reactions were negative.

The detection of insulin antibodies in diabetic patients

The results presented in Table II were obtained by using insulin-sensitized rabbit erythrocytes. The insulin-sensitized rabbit erythrocytes were used because they were agglutinated at higher dilutions of antiserum than the insulin sensitized sheep erythrocytes (19).

The sera of 23 diabetic patients were tested by the hemagglutination technique for insulin antibodies. The sera of eleven of the 23 diabetics contained antibodies to insulin. Seven patients re-

TABLE II

Antibodies to insulin in diabetic patients *

Patients	Insulin requirement *Units/day*	Antibody titre	Titre after inhibition with 10 μg. insulin	Comments
Ber.	2,000	1/160	0	Patients whose sera contained antibodies to insulin.
R. S.	2,000	1/64	1/8	
Weil.	600	1/1280	1/20	
Hen.	500	1/32	1/4	
O. S.	500	1/64	1/16	
O. H.	125	1/160	1/20	
J. G.	120	1/128	1/16	
D. S.	90	1/128	1/16	
B. B.	85	1/40	0	
Sed.	70	1/12	1/3	
Cug.	40	1/160	1/10	
A. S.	Diet only	1/32	1/16	Patients with questionable presence of antibodies.
J. D.	40	1/16	1/8	
D. R.	65	1/16	1/8	
L. S.	100	0	0	Patients whose sera did not contain antibodies to insulin.
A. C.	45	0	0	
H. S.	40	0	0	
N. F.	25	0	0	
Wil.	5	1/8	1/8	
H. S.	Diet only	0	0	
W. J.	No insulin	0	0	
Bur.	No insulin	0	0	
Jef.	New diabetic	0	0	

* The authors wish to thank the following persons who graciously made available the sera of the diabetic patients: Dr. James Craig of the Department of Medicine, Western Reserve University, Cleveland, Ohio; Dr. E. Perry McCullagh, Cleveland Clinic; Dr. Mary Loveless, N. Y. Hospital, New York City; Dr. Bernard Charms of Mount Sinai Hospital, Cleveland, Ohio.

ceived 120 to 2000 units of insulin per day. Antibodies to insulin were found in the sera of each of these seven patients. Four other patients who had antibodies to insulin received 70 to 90 units of insulin per day. Antibodies to insulin were not detected in nine of the patients. Four of these had never received insulin. One patient in whom antibodies were not detected received 100 units of insulin per day. The presence of antibodies in the sera of the remaining three patients was questionable.

Insulin assay

Where a semi-quantitative and rapid estimate of insulin content was desired, the hemagglutination method was used.[3] Known amounts of insulin were preincubated with each of several serial dilutions of a single insulin antiserum. In the control, saline was preincubated with identical dilutions of the same antiserum. The methods and materials used in this case were the same as described for the hemagglutination titration of an antiserum earlier in this paper.

The antiserum titre was decreased as increasing amounts of insulin were preincubated with the antiserum. Because this method was only semi-quantitative and the end point of agglutination was to a large extent subjective, the parameters of the method were not investigated thoroughly.

Therefore, it was decided to investigate an immune hemolysis method for assaying insulin. It was found that the amount of lysis of insulin-sensitized erythrocytes by a given dilution of antiserum varied inversely as the logarithm of the amount of insulin preincubated with the antiserum (Figure 3).

The methods and materials including final volume used in the hemolytic assay were the same as in the hemolytic titration as described earlier in this paper. The dilution of any given antiserum used in the assay was determined first by doing a hemolytic titration (Figure 2). Those dilutions where the inhibition of lysis of insulin-sensitized cells was maximal when 10 μg. of insulin was preincubated with the antiserum, were used in establishing standard curves for the antiserum involved (Figure 2). Various amounts of insulin in equal volumes (0.1 ml.) were preincubated with a given dilution of antiserum prior to the addition of complement and insulin-sensitized erythrocytes. The amount of hemoglobin released into the super-

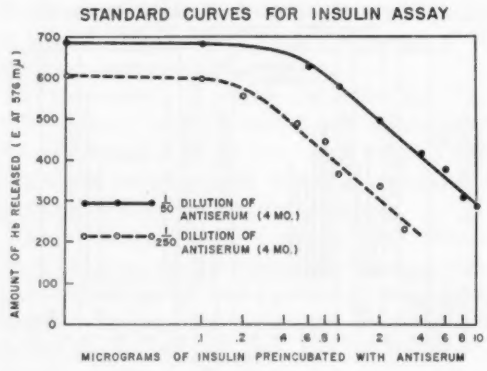

FIGURE 3

[3] This method has been used previously in this laboratory (8) for determining the concentration of egg albumin in egg white and the amount of diphtheria toxin or toxoid remaining in the supernatant in the quantitative flocculation reaction with antitoxin.

natant (within certain limits) diminished as a straight line function of the log of the amount of insulin preincubated (Figure 3). As the dilution of antiserum was increased, the standard curve shifted to the left. Consequently, the sensitivity of the method was dependent upon the dilution of the antiserum.

In Figure 3, typical standard curves of fifty times and two hundred fifty times diluted insulin antiserum are presented. When 1 to 10 μg. of insulin were preincubated with the 1/50 (rabbit 4 MO.) antiserum, the inhibition of lysis of insulin-sensitized erythrocytes was linear. When 0.2 to 5 μg. of insulin were preincubated with the same antiserum diluted 1/250, the curve was also linear. Samples containing between 1 and 10 μg. of insulin in 0.1 ml. were assayed using the fifty times diluted antiserum. Those containing below a microgram of insulin were assayed with 1/250 dilution of antiserum. There was about a fifteen per cent error when duplicates of the same point of the standard curve were run.

Complement was used in excess (0.5 ml. of 1/10 guinea pig serum) so that the amount of antibody not bound by insulin during preincubation and hence available for hemolysis was the only limiting factor. The factors limiting the sensitivity of the assay are discussed below. Thus far the assay has been applied only to the measurement of insulin in solution.

DISCUSSION

Previous evidence (12–15) indicates that insulin is weakly antigenic. By use of serological methods antibodies to insulin could not be demonstrated in all immunized animals. Even by use of highly sensitive methods such as anaphylaxis (24) insulin antibodies have not been detected in all immunized animals. Nor have insulin antibodies been demonstrated in all insulin-resistant diabetics (25).[4]

Borduas and Grabar (6) and Stavitsky (7) have presented evidence for the superior sensitivity of the hemagglutination compared to other serological and immunological methods. With these techniques insulin antibodies were demonstrated in virtually every immunized animal and in seven patients requiring 120 to 2000 units of insulin daily.

The sera of rabbits immunized with alum-precipitated crystalline beef insulin exhibited many properties of *in vivo* and *in vitro* antigen-antibody reactions. These antisera agglutinated insulin-conjugated rabbit or sheep erythrocytes. This agglutination was inhibited by preincubation of insulin with these antisera. The insulin antisera did not agglutinate non-sensitized sheep or rabbit erythrocytes. The antiserum lyzed insulin-sensitized red cells in the presence of complement. This hemolysis was inhibited by preincubation of insulin with the antiserum. The insulin antibodies were found in the gamma globulin fraction of the rabbit serum.[5] An anamnestic response to a single injection of insulin was elicited in rabbits which were previously immunized. In the next paper (16) data are presented concerning the serological cross-reactivity of insulins from various species, the neutralization of the physiological effects of insulin, and the specificity of the insulin antibodies produced in the rabbit.

The failure to observe ring and precipitin reactions with the sera of intensively immunized rabbits may be explained in one or two ways. First, the antibodies may not have been present in amounts sufficient to form visible aggregates with the amounts (38 to 300 micrograms) of antigen employed. This is a good possibility since even 38 micrograms may constitute a large excess of antigen for all but potent antisera. Therefore, when larger amounts of serum are available they should be tested for precipitins with amounts of insulin from 0.1 to 2 or 3 micrograms using 1 ml. samples of serum, and allowing the tests to remain in the refrigerator for a week before centrifuging and reading. The second possibility is that there may have been produced antibodies of a type which could be detected by the hemagglutination and hemolytic but *not* by the ring and precipi-

[4] Since the completion of this study Moloney and Coval (26) have presented new evidence for the antigenicity of insulin. Insulin-neutralizing antibodies for insulin were induced in guinea pigs and in sheep by injections of insulin in Freund's adjuvant. Mice were rendered diabetic by injection of guinea pig anti-insulin serum.

[5] Since the completion of this study Sehon, Kaye, McGarry, and Rose (27) by zone electrophoresis have isolated an insulin-neutralizing factor in the gamma globulin fraction of the serum of an insulin-resistant patient. They did not observe precipitin reactions between this serum or fraction and insulin.

tation methods. This is considered unlikely since it has been found (19) that non-precipitating antibodies do not hemagglutinate red cells treated with the homologous protein.

In view of the ability to demonstrate insulin antibodies in immunized rabbits and insulin-resistant diabetics, it is important to learn whether these antibodies affect the physiological action of endogenous or injected insulin. Berne and Wallerstein (25) have reviewed the work from many laboratories and concluded that the presence or absence of antibodies in insulin-allergic or insulin-resistant patients was not correlated with the degree of allergic response or resistance to insulin. However, insulin-neutralizing substances have been demonstrated in the serum of insulin-resistant patients and immunized rabbits. Lowell (28) using the blood sugar curve method in mice, has shown the presence of insulin-neutralizing substances in the blood of insulin-resistant diabetics. Recently, Marsh and Haugaard (29) observed a significant decrease in the amount of insulin that combined with a diaphragm preparation when the insulin was preincubated with sera from normal diabetic patients. They attributed this to substances in serum which combine with or inactivate insulin. Serum from insulin-resistant patients contained greatly increased amounts of these substances compared to normal, and non-resistant diabetics. We have evidence (16) indicating that the serum of insulin-immunized rabbits can neutralize the physiological effects of insulin as tested by the mouse convulsion method.

It may be significant that all seven patients who required between 120 and 2,000 units of insulin per day had antibodies to insulin. However, it is not known whether the presence of antibodies accounted for the high daily requirement or whether the antibodies resulted from the daily injection of large amounts of insulin.

The hemagglutination method may facilitate the study of the immunological aspects of insulin resistance. However, it is probable that only antibodies uncombined with insulin can be detected. Therefore, in patients exhibiting low titres it may be necessary to withdraw insulin for several days before antibodies become apparent.

With the hemagglutination and hemolytic methods it has been possible to detect 0.1 microgram of insulin in solution. It is felt that with further work, the assay can be made more sensitive. The biological methods have been able to detect as little as 0.001 microgram of insulin.

Anderson, Linder, and Sutton (2) was able to measure 0.02 milliunits (about 0.001 μg.) using adrenal demedullated, alloxan diabetic, hypophysectomized rats. Bornstein (3) using hypophysectomized, alloxan diabetic, and adrenalectomized rats has been able to detect 0.05 milliunits (about 0.002 μg.). Groen, Kamminga, Willebrands, and Blickman (4) assayed insulin by its effect on the glucose utilization of the isolated rat diaphragm. They presented evidence in rough agreement with Anderson, Linder, Sutton, and Bornstein as to the level of insulin in serum.

The biological methods do not measure insulin itself, but only its effects. Therefore, these assays are useful in measuring insulin in samples which do *not* contain any substances that affect glucose metabolism. Unfortunately, many tissues and biological fluids may contain substances, such as adrenaline and hormones of the adenohypophysis which do affect glucose metabolism. These methods also show considerable biological variation and require a large number of experimental procedures and statistical analysis to establish their validity and reliability.

The reliability of the immunological assay of insulin depends particularly upon the specificity of the antiserum. In the following paper (16) evidence is presented for the specificity of the rabbit antibodies for insulin itself, and for the immunological similarity of insulins from various species. Therefore, the hemolytic method employing rabbit antiserum to beef insulin is probably applicable to the measurement of insulin from various species.

The sensitivity of the immune hemolysis assay for insulin is dependent upon the dilution of antiserum employed. This is limited, because as the dilution of the antiserum increases, the curve becomes flattened and consequently the error greater. The assay of insulin by this method is also limited by a high non-specific lysis of erythrocytes. This non-specific lysis is dependent upon a heat labile substance in guinea pig serum which, in the presence of complement, causes the lysis of both non-sensitized and insulin-conjugated sheep erythrocytes. It appears that this substance is similar in

many respects to properdin recently described by Pillemer and his associates (30). Studies on the removal of properdin from guinea pig serum with sheep erythrocyte stromata will be presented elsewhere (31). The effect of properdin on the hemolytic assay of proteins is being investigated.

It is not known at present whether the hemolytic assay will distinguish between physiologically active and inactive insulin. Also unknown is the applicability of the assay to the measurement of insulin in tissues and fluids.

SUMMARY

1. Methods are presented for the conjugation of insulin to sheep erythrocytes, with bis-diazobenzidine.

2. Antibodies to insulin can be consistently detected in rabbits using hemagglutination or hemolysis of the insulin-conjugated sheep erythrocytes as an end point.

3. Repeated intravenous injections of adequate amounts of alum-precipitated insulin over a sufficiently long period of time can consistently elicit antibodies to insulin in the rabbit.

4. Evidence is presented for the suggestion that the hemagglutination of insulin-conjugated erythrocytes by the sera of diabetic patients may be of value for future study of the immunological aspects of insulin resistance.

5. Methods are presented for the assay of microgram quantities of insulin in pure solutions.

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EVIDENCE FOR THE INSULIN-DIRECTED SPECIFICITY OF RABBIT ANTI-INSULIN SERUM [1]

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(Submitted for publication October 31, 1955; accepted December 28, 1955)

In the previous paper (2) a method was described for the immunological assay of microgram quantities of insulin. This method employed the reaction between rabbit antiserum to insulin and sheep erythrocytes conjugated with insulin. However, the applicability of the method to the measurement of insulin is obviously dependent upon the insulin-directed specificity of the antiserum which is employed in the assay. Therefore, the present experiments were designed to determine the specificity of rabbit antisera to crystalline beef insulin. In order to establish this specificity answers were sought to these questions: 1) To what extent will the insulin antisera react with contaminants that may be present in the crystalline beef insulin used for immunization? 2) Will these antisera react with proteins derived from beef plasma and other sources? 3) To what extent will the antisera to beef insulin react with insulin from other species? 4) Will the antisera interfere with the physiological action of insulin?

Previous investigators have provided much information on some of these points. Lewis (3) and Barral and Roux (4) have shown that purified insulin is a different antigen from those in serum or crude extracts of pancreas. The immunological similarity of insulin prepared from several species has been demonstrated by Lewis (3) and also by Wasserman and Mirsky (5). Using the Schultz-Dale technique, Lewis (3) showed that guinea pigs can be sensitized with insulin from one species and shocked with insulin from another species. Wasserman and Mirsky (5) confirmed this in sensitization and complement fixation experiments. However, these experiments were qualitative in nature. Furthermore, the insulin preparations employed were less pure than those used in the present study.

The ability of insulin antibodies to neutralize the physiological action of insulin has been demonstrated by Lowell (6), Banting, Franks, and Gairns (7) and Marsh and Haugaard (8). Recently, Moloney and Coval (9), using insulin neutralization techniques, have demonstrated the immunological similarity of crystalline insulin from several species. He was also able to produce diabetes in the mouse by the intravenous injection of guinea pig antiserum to insulin.

The present experiments were performed with rabbit insulin antisera. The hemolysis-inhibition method described in the previous paper (2) was employed in testing the specificity of these antisera, because this is the method which is proposed for the assay of insulin.

The evidence obtained indicates that these antisera are highly specific for insulin *per se*. The results also show that these antisera cross-react to a great extent with insulin from four species. Evidence for the interference of rabbit insulin antiserum with the physiological action of insulin in mice is also presented.

MATERIALS AND METHODS

Antigens

Crystalline beef insulin (lot No. 535664). [3] This is a sample which contains 27 units per mg. There is some hyperglycemic factor present in the preparation. This sample was used as the antigen in preparing the antisera (2).

Trypsinized crystalline beef insulin (lot No. 466368). [3] This sample contains no hyperglycemic activity and assayed at 27 units per mg. It probably contains traces of trypsin.

Human insulin. [4] This sample contained cresol as preservative and assayed at 11 units per ml.

[1] This study was supported in part by a grant from the Eli Lilly Co. A preliminary report of this work was presented before the American Association of Anatomists in Philadelphia in the Spring of 1955 (1).

[2] This study was conducted in part during the tenure of an Established Investigatorship from the American Heart Association.

[3] We wish to thank the Eli Lilly Co. for the beef insulin samples, the bovine glucagon, and the crude extract of beef pancreas.

[4] We wish to thank the Connaught Research Laboratories for the solution of human insulin.

The "A" components of sheep, pork, and beef insulin. Using the counter-current distribution technique Harfenist and Craig (10) have been able to separate supposedly homogeneous insulin samples into two components, A and B, with distinctly different partition ratios. The material recovered from each peak was crystalline and both of about equal biological activity. The only difference between the A and B components is that the former contains six amide groups whereas the latter contains only five. These "A" components of sheep, beef, and pork insulin were prepared by Harfenist and Craig and graciously presented to us.

Crude extract of beef pancreas.[5] This preparation was assayed by the mouse convulsion method and contained 0.5 units of insulin per mg. of dry weight.

Bovine glucagon. This is a highly purified preparation of crystalline glucagon (hyperglycemic factor).

Other proteins. Bovine gamma globulin lot No. C904 (Armour Co.) is fraction II of bovine plasma. Bovine albumin lot No. L11705 (Armour Co.) is fraction V of bovine plasma. Egg albumin lot No. E90115 (Armour Co.) is a 3 times recrystallized preparation. Human gamma globulin[5] from plasma is fraction II, 1, 2, which was prepared by the Cohn method IX (11). Human serum albumin[5] from plasma is fraction V which was prepared by the Cohn method VI (12).

Insulin conjugated erythrocytes. The preparation of these cells, including the conjugating agent, bis-diazobenzidine, was described in the previous paper (2).

Sera

Rabbit sera were employed as a source of diluent for the antiserum as well as of antibodies to insulin and egg albumin. The insulin antisera were produced in adult white rabbits using alum-precipitated beef insulin (lot No. 535664), as described in the previous paper (2). The egg albumin antisera were prepared as previously described (13).

The preparation of absorbed guinea pig serum (source of complement). The sensitivity of the methods for the hemolysis titration of insulin antiserum and the hemolytic assay for insulin as described in the previous paper (2) was limited by the high background due to nonspecific hemolysis of the sheep erythrocytes by guinea pig serum. It was observed in our laboratories and independently by Brumfield and Ross (14) that guinea pig serum in high concentration causes hemolysis of either insulin-conjugated or non-conjugated sheep erythrocytes. For instance, about 0.06 ml. of pooled guinea pig serum is capable of causing complete hemolysis of 0.1 ml. of a 2 per cent suspension of nonsensitized sheep erythrocytes in a final volume of 0.6 ml. In order to avoid this nonspecific hemolysis the guinea pig serum used as the source of complement is absorbed with an equal volume of washed packed sheep erythrocytes for 1 hour at 10°C. After absorption the complement titre decreases about 20 per cent probably because it is diluted during the absorption. The factor responsible for the nonspecific hemolysis is decreased from 16 units per ml. (0.06 ml. guinea pig serum to 0.1 ml. erythrocytes) to less than 2 units per ml. Evidence indicating that this factor is properdin has been presented in another paper (15).

Titration of antiserum by hemolysis inhibition. In testing the specificity of the insulin antisera against various substances the method for the hemolytic titration of rabbit antiserum to insulin which has been described in the previous paper (2) was used. Serial dilutions of a given rabbit antiserum were prepared in 1/100 normal rabbit serum. In the control serial dilutions, saline (0.1 ml.) was preincubated with each dilution. In the experimental serial dilutions the test material in 0.1 ml. was preincubated with each dilution. The amount of hemolysis of the insulin conjugated erythrocytes at each dilution in the control set of serial dilutions was compared with the amount of hemolysis obtained in each dilution of the experimental sets of serial dilutions. In this way it was possible to determine to what extent any given protein or insulin sample reacted with the antiserum. The ability of the test protein or insulin to react with the rabbit antiserum to crystalline beef insulin was manifested by the extent to which hemolysis was inhibited.

The neutralization of insulin by rabbit antiserum in the mouse. The ability of mice to remain clinging to a wire mesh after they had been injected with insulin (lot No. 535664) which had been mixed with either rabbit antiserum or normal rabbit serum, was used to test the capability of antiserum to neutralize the insulin.

The mice were injected with 0.4 μg. of crystalline beef insulin per gram body weight, which was equivalent to 0.1 unit per gram body weight. The insulin was preincubated with either insulin antiserum or normal rabbit serum for more than 5 minutes but less than 15 minutes prior to injecting the mice with the mixture of serum and insulin. The preincubation mixture consisted of a ratio of 2 ml. of serum to 0.5 ml. of insulin solution containing 100 micrograms of insulin. One ml. of this mixture was equivalent to 1.04 units of insulin. The mice were selected at random for the control and experimental group using a table of random numbers from Snedecor (16). The mice were injected intraperitoneally with 1 ml. (per 10 grams body weight) of the mixture containing either antiserum and insulin or normal rabbit serum and insulin. They were then placed on a ¼ inch wire mesh screen which was inclined at an angle of 70 degrees. The length of time they remained clinging to the screen was noted.

RESULTS

Reactions between rabbit insulin antiserum and other pancreatic proteins

Glucagon. Glucagon, which is a known contaminant of crystalline insulin preparations, was tested in two systems to determine whether in-

[5] We wish to thank Drs. Louis E. Pillemer and Irwin Lepow for the preparations of human gamma globulin and human serum albumin.

hibition of hemolysis was associated with a specific reaction between glucagon and an antibody in the insulin antiserum. Egg albumin sensitized cells and rabbit egg albumin antiserum were employed in the control system. To insure the presence of sufficient antigen to react with antibody to glucagon in the insulin antiserum, hemolysis-inhibition by *100* μg. of beef glucagon was compared with that by *10* μg. of crystalline beef insulin.

Addition of glucagon caused the same small decrease of hemolysis in both the insulin and egg albumin systems as the addition of saline (Figure 1), indicating non-specific inhibition of hemolysis rather than a reaction of glucagon with antibody. On the other hand, 10 μg. of insulin were considerably more effective than 100 μg. of glucagon in specifically decreasing the hemolysis in the insulin system.

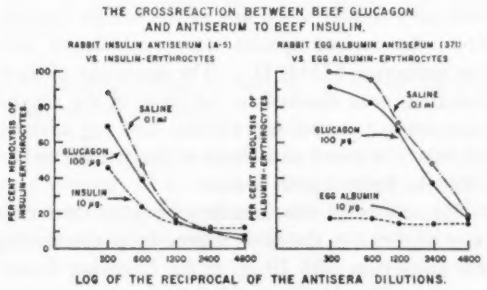


FIGURE 1

Crude bovine pancreatic extract. If the rabbit insulin serum is indeed specific for insulin *per se*, it should react only with the insulin that is present in a crude fraction of pancreas. Therefore, the ability of 100 μg. of crude pancreatic extract (containing 0.5 units of insulin per mg. dry weight) to react with insulin antiserum, was compared with that of 1.8 μg. crystalline insulin containing an equivalent number of units. These calculations were based on the unitage of crystalline beef insulin (*27* units per mg.).

The inhibition of hemolysis was comparable when either the crude pancreatic extract or the crystalline insulin was preincubated with the rabbit insulin antiserum (Figure 2). If contaminants of insulin had combined with the antiserum, inhibition of hemolysis by the pancreatic extract should have been much greater than that observed with

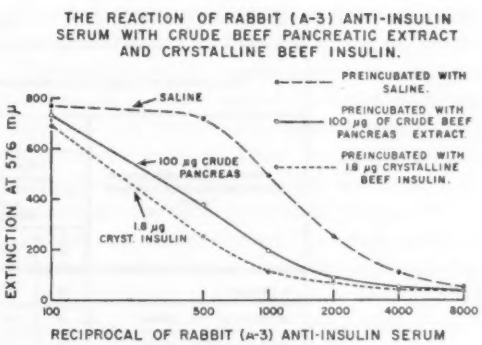


FIGURE 2

an equivalent number of units of crystalline insulin. Therefore, with this method antibodies to bovine pancreatic contaminants of insulin could not be detected in rabbit antiserum to crystalline beef insulin.

Reactions between insulin antisera and foreign proteins

Experiments were designed to detect reactions between rabbit insulin antisera and any of the following proteins: egg albumin, human serum albumin, human gamma globulin, bovine serum albumin, and bovine gamma globulin (Table I).

The titration curve obtained when each of these proteins was preincubated with the insulin antiserum was very similar to that obtained when saline was preincubated with the same dilutions of antiserum. On the other hand, there was a marked inhibition of hemolysis of the insulin-conjugated erythrocytes by preincubation of crystalline insulin with the antiserum (Table I).

There was, therefore, no observable reaction be-

TABLE I

THE CROSSREACTIVITY OF RABBIT ANTI-INSULIN SERUM WITH PROTEINS OTHER THAN INSULIN.

PROTEINS (10 µg) PREINCUBATED WITH THE ANTISERUM	DILUTIONS OF INSULIN ANTISERUM				
	1/75	1/150	1/300	1/600	1/1200
	PER CENT HEMOLYSIS OF INSULIN-ERYTHROCYTES				
0.1 ml SALINE	92	80	56	30	18
EGG ALBUMIN	88	80	52	29	16
HUMAN SERUM ALB.	87	78	51	22	17
HUMAN GAMMA GLOB.	88	81	57	33	20
BOVINE SERUM ALB.	87	84	57	33	20
BOVINE GAMMA GLOB.	92	88	60	36	20
INSULIN LOT #535664	19	18	14	11	10

TABLE II

THE REACTION OF RABBIT ANTI – INSULIN SERUM WITH INSULIN FROM VARIOUS SPECIES

INSULIN (TEN MICROGRAMS) PREINCUBATED WITH THE ANTI SERA	PERCENT HEMOLYSIS							
	INSULIN CONJUGATED SHEEP ERYTHROCYTES				CRYSTALLINE EGG ALBUMIN CONJUGATED SHEEP ERYTHROCYTES			
	RABBIT (4MO) INSULIN ANTISERUM				RABBIT 371 EGG ALBUMIN ANTISERUM			
	1/75	1/150	1/300	1/600	1/400	1/800	1/1600	1/3200
0.1 SALINE	92	80	58	30	100	92	52	9
INSULIN – LOT NO. 535864	19	18	14	11	100	94	53	10
INSULIN – LOT NO. 448368	13	14	13	13	100	92	53	8
INSULIN (PORK) – (A) COMPONENT	16	12	12	11	100	100	54	8
INSULIN (SHEEP) – (A) COMPONENT	14	13	11	10	100	98	48	9
INSULIN (BEEF) – (A) COMPONENT	12	15	11	12	100	96	60	12
INSULIN (HUMAN)	24	22	14	12	100	88	88	23

tween the insulin antiserum and five proteins other than insulin, two of which were bovine in origin as was the insulin used to immunize the rabbits.

The cross-reactivity of rabbit insulin antiserum with highly purified samples of insulin from various species

Various insulin preparations were preincubated with rabbit insulin antisera before the addition of sheep erythrocytes conjugated with crystalline beef insulin. The degree of inhibition of hemolysis was very similar when any of the insulin samples from the various species was preincubated with the antiserum (Table II). The specificity of these reactions was checked by addition of the insulin samples to a control egg albumin-anti egg albumin system. In every case these additions did not inhibit the heterologous system.

Hemolysis of the insulin-conjugated red cells was inhibited to the same degree by preincubating the antiserum with 10 μg. of the following insulin samples: The highly purified "A" components of beef, sheep, and pork insulin or crystalline beef and trypsinized beef insulin. When these samples were incubated with the 1/75 dilution of the antiserum, 12 to 19 per cent hemolysis was observed. When saline was preincubated with the 1/75 antiserum dilution 92 per cent hemolysis was observed (Table II). Human insulin also inhibited hemolysis when preincubated with insulin antiserum. More hemolysis (less inhibition) was observed with human insulin than with an equal amount of any of the other insulin samples. However, an increased hemolysis of egg albumin conjugated erythrocytes, greater than that seen with egg albumin antiserum dilutions preincubated with saline, also observed when human insulin was tested in the egg albumin system. This non-specific hemolysis of the erythrocytes may have been the result of the cresol present in the human insulin solution (Table II).

The extent to which insulin antisera hemolyzed

CROSS REACTIONS OF RABBIT ANTI BEEF INSULIN SERUM (LILLY'S CRYSTALLINE ZINC) WITH SHEEP ERYTHROCYTES CONJUGATED WITH THE "A" COMPONENT OF INSULIN PREPARED BY COUNTER CURRENT DIFFUSION.

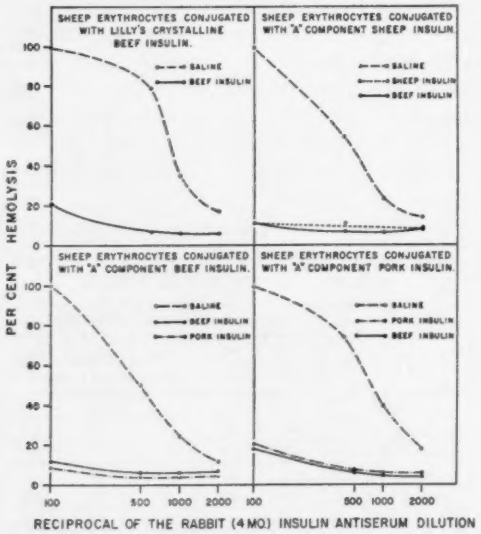


FIGURE 3

erythrocytes conjugated with "A" components of insulin was also tested (Figure 3). Cross-inhibition experiments, in which these "A" components of sheep, pork, beef insulin, and crystalline beef insulin were preincubated with the insulin antiserum, were simultaneously performed. Figure 3 demonstrates the similarity of the hemolytic titration curves obtained when rabbit insulin antiserum was mixed with erythrocytes conjugated with "A" components of sheep, pork, or beef insulin. The "A" components of beef insulin inhibited the hemolysis of cells conjugated with sheep, pork, or beef insulin. The "A" component of sheep insulin inhibited the hemolysis of red conjugated with sheep insulin. The "A" component of pork insulin inhibited the hemolysis of cells conjugated with beef and pork insulin. Thus, it is apparent that rabbit antiserum against crystalline beef insulin cross-reacts to a high degree with the "A" components of sheep, pork, and beef insulin.

The neutralization of insulin by rabbit insulin antisera in the mouse

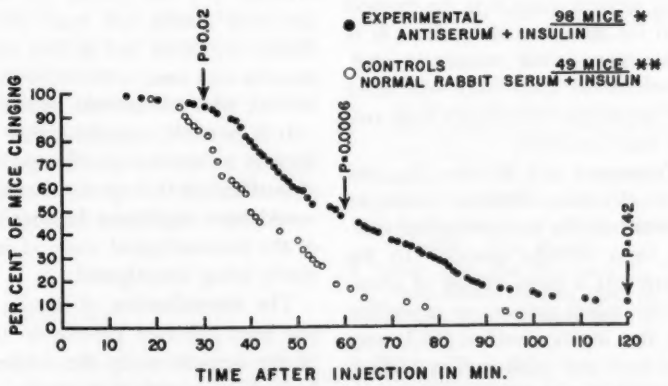
Insulin may be measured and characterized by its physiological action, *i.e.*, the lowering of blood sugar and the subsequent production of insulin shock. In the characterization of any insulin antiserum it is, therefore, desirable to demonstrate the *in vivo* neutralization of insulin by the antiserum.

Solutions of rabbit insulin antiserum incubated with insulin at room temperature prolonged the length of time mice could maintain their body weight on an inclined screen as compared with solutions of normal rabbit serum incubated with insulin under the same conditions (Figure 4).

Insulin was mixed with normal rabbit serum or antiserum not less than five minutes or longer than 15 minutes before these solutions were injected intraperitoneally into mice. Ninety-eight experimental mice were injected with the mixture of antiserum and insulin and forty-nine control mice were injected with the mixture of normal rabbit serum and insulin. The mice injected with a mixture of antiserum and insulin remained clinging to the screen significantly ($p - 0.0004$) longer than the mice injected with the mixture of normal rabbit serum and insulin.

Mice which were injected with a mixture of insulin and normal rabbit serum which had incubated at room temperature longer than 15 minutes were able to hang on to the screen significantly ($p - 0.04$) longer than those mice which were injected with a mixture of insulin and antiserum which had been incubated a comparable length of time (Figure 5).

INSULIN ANTISERUM PROTECTION AGAINST INSULIN IN THE MOUSE.



* THE EXPERIMENTAL MICE WERE INJECTED INTRAPERITONEALLY WITH 0.4 MICROGRAMS OF CRYSTALLINE INSULIN /GM BODY WEIGHT. THIS INSULIN HAD BEEN PREVIOUSLY MIXED WITH RABBIT INSULIN ANTISERUM (LESS THAN 15 MINUTES). 1 ML /50 GAMMAS OF INSULIN EQUIVALENT TO 1 ML /1.04 UNITS.

** THE CONTROL MICE WERE TREATED THE SAME AS THE EXPERIMENTAL EXCEPT THAT NORMAL RABBIT SERUM WAS USED INSTEAD OF INSULIN ANTISERUM.

FIGURE 4

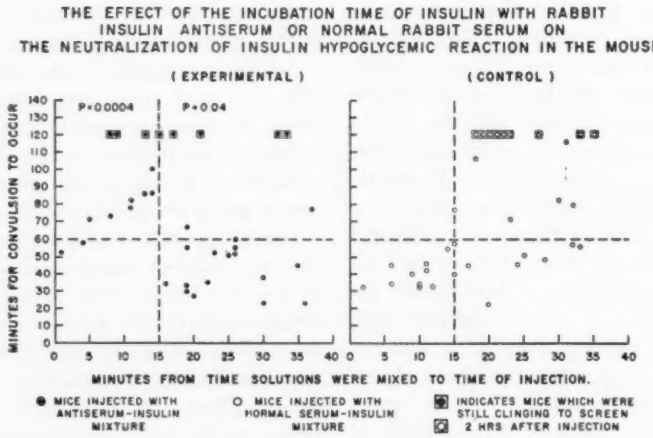

THE EFFECT OF THE INCUBATION TIME OF INSULIN WITH RABBIT INSULIN ANTISERUM OR NORMAL RABBIT SERUM ON THE NEUTRALIZATION OF INSULIN HYPOGLYCEMIC REACTION IN THE MOUSE.
(EXPERIMENTAL)
(CONTROL)
P=0.0004
P=0.04
MINUTES FOR CONVULSION TO OCCUR
MINUTES FROM TIME SOLUTIONS WERE MIXED TO TIME OF INJECTION.
MICE INJECTED WITH ANTISERUM-INSULIN MIXTURE
MICE INJECTED WITH NORMAL SERUM-INSULIN MIXTURE
INDICATES MICE WHICH WERE STILL CLINGING TO SCREEN 2 HRS AFTER INJECTION

FIGURE 5

DISCUSSION

Using the hemolytic titration of rabbit insulin antiserum, as described in the previous paper (2), evidence has been obtained that the rabbit antisera to insulin are highly specific for insulin: 1) There was no observable reaction with foreign proteins. 2) There was little if any reaction with antigens from bovine serum or bovine pancreas. 3) The antisera reacted quantitatively with only the insulin in a crude extract of pancreas. 4) The insulin-induced hypoglycemic reaction was delayed by the rabbit insulin antisera in the mouse.

The hemolytic method was used to test the specificity of these antisera since it is the method which is proposed for the assay of insulin. It is not known whether these rabbit antisera to insulin would have reacted so specifically had other immunological or serological techniques been employed in testing their specificity.

Lewis (3), Wasserman and Mirsky (5), and Moloney and Coval (9) using different techniques were able to demonstrate the immunological similarity of insulin from various species. In the present study there was a great degree of cross-reaction between the rabbit antisera to crystalline beef insulin and the highly purified "A" components of sheep, beef and pork insulin. However, these results do not mean that insulins from various species are identical. These results are in accord with the finding by Sanger (17) that insulin preparations from ox, sheep and pork are very similar but *not* identical in amino acid composition.

Whether these antisera will react with the endogenous insulin in the various species tested is difficult to predict. There does not seem to be any indication that the antibodies produced by the rabbit in any way reacted with the endogenous insulin of the rabbit. Repeated glucose tolerances and periodic blood glucose determinations of the rabbits in various phases of immunization were consistently normal. This has also been observed by Moloney and Coval (9) in sheep and guinea pigs. Moloney and Coval have also presented evidence (9) that guinea pig antisera to crystalline pork insulin will react with "altered" pork, sheep, ox, rabbit and guinea pig insulin. These antisera also react with endogenous mouse insulin but not with endogenous guinea pig insulin.

It is possible, therefore, that endogenous insulin may be species specific and that in the course of purification this species specificity is lost. This would have significant bearing on the application of the immunological assay of insulin and is currently being investigated.

The neutralization of insulin by immune sera has been reported previously (6–9). However, in the present study the antiserum delayed the hypoglycemic action of insulin only when mixed with insulin for less than fifteen minutes. Under these conditions, the immune serum neutralized insulin rapidly compared to normal rabbit serum.

Mice injected with solutions of normal rabbit serum and insulin mixed longer than 15 minutes clung to the screen longer than those mice which which were injected with solutions of immune rabbit serum and insulin which had been incubated longer than 15 minutes. Mice injected with solutions of insulin and normal rabbit serum incubated longer than 15 minutes clung to the screen longer than mice injected with the same solutions which were incubated less than 15 minutes. Therefore, normal serum alone delayed the hypoglycemic action of insulin when mixed with insulin longer than 15 minutes at room temperature. This delayed inhibition of insulin was not observed when the immune serum was incubated with the insulin at room temperature longer than 15 minutes.

Explanation of the transitory nature of the neutralization of the immune serum on insulin by dissociation of the antibody antigen complex is presently without basis since no evidence of dissociation was noted *in vitro* by the hemagglutination or hemolytic methods.

The action of the normal rabbit serum (heated at 56°C for 30 minutes) on insulin is also difficult to explain; whether the insulin is destroyed or neutralized in some way by the normal rabbit serum is unknown.

The transitory neutralization of insulin by rabbit antiserum and the effect of normal rabbit serum on insulin have not been previously described. Therefore, it is not known to what extent these phenomena apply to the sera of other species, especially man. Certainly, if they apply to human sera, failure to take them into consideration might lead to erroneous conclusions.

On the basis of these observations, the hemolytic method employing rabbit antiserum to insulin appears to be specific and sensitive enough for the assay of insulin in biological fluids and tissues. This application is, therefore, being currently investigated.

SUMMARY

1. As tested by the immune hemolysis method, rabbit antisera to alum precipitated crystalline bovine insulin were highly specific for insulin.

2. These antisera cross-reacted to very marked degree with trypsinized beef insulin, human insulin, and the "A" components of sheep, pork and beef insulin.

3. As compared with normal rabbit serum, the rabbit insulin antisera significantly delayed the hypoglycemic action of insulin only when mixed with insulin for less than 15 minutes. Normal serum alone delayed the hypoglycemic action of insulin when mixed with insulin longer than 15 minutes.

4. In view of the evidence presented the application of these antisera to the assay of insulin in mixtures of proteins was suggested.

ACKNOWLEDGMENTS

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A STUDY OF THE RELATIVE BINDING CAPACITY OF PLASMA PROTEINS, INTACT HUMAN RED CELLS, AND HUMAN RED CELL STROMA FOR RADIOACTIVE I-131 LABELED L-THYROXINE

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Previous studies (1–3) have demonstrated the binding properties of serum protein for L-thyroxine and to a lesser extent for L-triiodothyronine. Previous studies from our laboratory (4) have shown that human red cells also have a binding or assimilating capacity for L-thyroxine and L-triiodothyronine.

The purpose of this paper is to present further information on the binding of L-thyroxine and L-triiodothyronine by human red cells. We will also present studies on the binding of these two substances by human red cell stroma, and the relative binding capacity for plasma proteins and human intact red cells.

METHODS AND MATERIALS

Whole blood, drawn with a heparinized syringe from healthy volunteers, was centrifuged in 15-ml. test tubes at 1500 rpm for 20 minutes. (The centrifuge radius was 18 cm. and the radial acceleration was 453 g's.) The plasma was drawn off and normal saline solution added to the tubes containing the packed red cells. These were then gently mixed and recentrifuged as before. The supernatant was again drawn off and the wash procedure repeated once more, the packed cells remaining being termed "washed red cells." In each washing sufficient normal saline solution was added to fill a 15 × 125 mm. test tube containing about 6 ml. of packed cells.

Test tubes containing 2 ml. of the radioactive standard, I-131-labeled sodium L-thyroxine or I-131-labeled sodium L-triiodothyronine, were set up and the amount of radioactivity of each in counts per minutes was determined in a well type (Nuclear R) scintillation counter. The standard was prepared by diluting 1 ml. of the radioactive L-thyroxine or L-triiodothyronine [2] 1 : 1000 with normal saline solution. This preparation is dissolved in 50 per cent propylene glycol. Aliquots of this dilution were then diluted from 2 to 100 times for the final standard depending on the amount of decay. This resulted in there being between 1.0×10^{-4} to 5.0×10^{-4} micrograms of hormone in 2 ml. of the standard. Two ml. of the washed cells were then added to each tube containing the precounted standard. These were gently mixed and incubated for 30 minutes at 37°C. After the incubation, normal saline solution was added to the tubes, the tubes gently mixed, centrifuged, and the supernatant drawn off. This procedure was repeated once more and the remaining 2 ml. of packed cells counted in a well counter. This counting rate was expressed as a percentage of the counting rate of the corresponding 2 ml. of radioactive standard. This percentage was termed the percentage uptake by the red cell.

After this procedure, 2 ml. of fresh human plasma was added to the same 2 ml. of red cells used for the uptake; the tubes gently mixed and incubated for 30 minutes at 37°C. The mixture was then centrifuged and the plasma drawn off. Following this the cells were washed twice with normal saline solution as after the uptake procedure. The counting rate at the end of this incubation and washing was then expressed as a percentage of the counting rate at the end of the uptake study. This was the percentage of the labeled material originally with the cell that remained with it after incubation with fresh plasma. The procedure was then repeated once more with another 2 ml. of fresh plasma to determine if further radioactivity could be removed from the cells. The variation in recovery of radioactivity as a function of the amount of plasma added was determined by preparing a series of eight tubes containing 0.0 to 0.50 ml. of plasma and made up to a volume of 2 ml. with normal saline solution. The contents of these tubes were then added to the cells remaining from the uptake study, gently mixed, incubated, and the washings repeated as above. The percentage of the radioactivity remaining with the red cells subtracted from one hundred gave the percentage of radioactivity with the cell recoverable by the plasma-saline mixtures.

Varying amounts of stable L-thyroxine were dissolved in 0.02 M NaOH solution so that each 0.1 ml. of the final solutions would contain the following micrograms of L-thyroxine: 2.5, 5, 10, 15, 20, 30, 50 and 100. Human fresh plasma (1.0 ml.) was then incubated with 0.1 ml. of each of the above solutions for 30 minutes at 37°C. Then 0.1 ml. of this "incubated" plasma was added to the radioactive L-thyroxine and L-triiodothyronine and allowed to stand for 15 minutes. Two ml. of

[1] S. E. Massengill Fellowship for student research during summer of 1955.

[2] Obtained from Abbott Laboratories, Oak Ridge, Tennessee.

washed human red cells were added to each tube and the uptake by the red cells of the radioactive labeled material determined as described previously (4). A control study was also carried out using 0.1 ml. of 0.02 M NaOH solution.

The uptake by twenty-one-day old refrigerated human washed red cells of radioactive labeled L-thyroxine and L-triiodothyronine was also carried out.

Human red cells were washed with a 0.02 M sodium fluoride solution allowing the sodium fluoride solution to remain in contact with the red cells for at least 30 minutes before centrifugation. The uptake by these cells for radioactive I-131 labeled L-thyroxine and L-triiodothyronine was determined in the usual manner.

The uptake by the human red cells of radioactive labeled L-thyroxine and L-triiodothyronine was carried out at the following temperatures: 0°, 5°, 15°, 25°, 30°, and 37° Centigrade.

Red cell stroma was prepared according to the method of Tishkoff, Robscheit-Robbins, and Whipple (5) who used a modification of the method of Ballentine (6). The uptake and recovery for the stroma was done much like that of the cells. Two ml. of the stroma suspension was suspended in 2 ml. of previously counted radioactive standard and incubated for 30 minutes at 37°C. In the case where human fresh plasma was added it was simply added to the radioactive standard solutions and allowed to stand for 15 minutes at 37°C before the stroma was added and incubated. After incubation the stroma was then washed twice with normal saline solution as in the cell study. Applicator sticks were used to resuspend the stroma after each centrifugation. This introduces a negligible error as no radioactivity remained with the applicator. The stroma was then counted after the last centrifugation and this expressed as the per cent uptake as in the cell study.

Recovery was done as in the cell study by the resuspension of the stroma (used for the uptake studies) in 2 ml. of fresh human plasma. Again applicator sticks had to be used to suspend the stroma. The recovery suspensions were incubated for 30 minutes at 37°C, washed twice as above, and recounted. The recovery was expressed as in the red cell study as a percentage of the count with the stroma at the end of the uptake study.

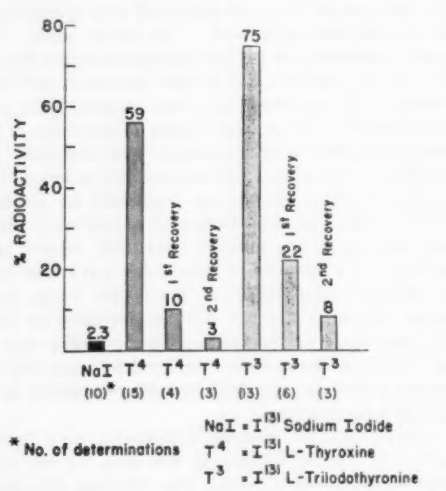

FIG. 1. THE UPTAKE BY HUMAN WASHED RED CELLS OF RADIOACTIVE I-131 L-THYROXINE, L-TRIIODOTHYRONINE, SODIUM IODIDE AND THE PER CENT OF RADIOACTIVITY REMAINING WITH THE RED CELL AFTER THE RECOVERY OF THE RADIOACTIVE LABEL BY SUCCESSIVE INCUBATIONS WITH FRESH HUMAN PLASMA

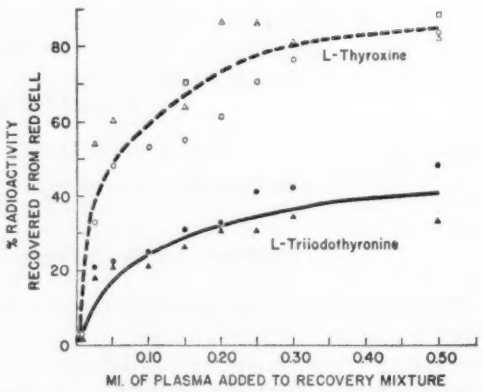

FIG. 2. THE RECOVERY OF RADIOACTIVE LABEL FROM HUMAN WASHED RED CELLS BY FRESH HUMAN PLASMA-SALINE MIXTURES

RESULTS

The average uptake by the washed human red cells of radioactive labeled L-thyroxine was 59 per cent (range 45 to 66 per cent) and for L-triiodothyronine was 75 per cent (range 68 to 80 per cent). It is to be emphasized again that by uptake is meant the per cent of radioactivity remaining with the red cells which cannot be removed by washing with normal saline. After incubation of the red cells (which have been used in the uptake study) with fresh human plasma for 30 minutes at 37°C all but 10 per cent (range 8 to 12 per cent) of radioactivity can be removed from the cells that had been previously incubated with radioactive L-thyroxine and all but 22 per cent (range 18 to 26 per cent) of the radioactivity from the cells previously incubated with radioactive L-triiodothyronine. Further incubation with fresh human plasma reduces the residual radioactivity to 3 per cent (range 2 to 6 per cent) for

the L-thyroxine incubated cells and to 8 per cent for L-triiodothyronine incubated cells. The recovery bars in Figure 1 indicate the percentage of previously retained radioactivity which now remains with the cells after one or two incubations with fresh human plasma (Figure 1).

The amount of radioactivity recovered from the tagged cells was increased as the amount of human fresh plasma with which they were incubated was increased from 0 to 0.5 ml. Human fresh plasma was more effective in removing the radioactive tag from the cells incubated with radioactive L-thyroxine than from those cells incubated with L-triiodothyronine (Figure 2). Normal saline solution was again ineffective in removing the radioactive tag from the incubated cells.

The findings using plasma incubated with stable thyroxine are plotted in Figure 3 along with the NaOH controls in which 0.10 ml. of 0.02 M NaOH was added per ml. of plasma, 0.10 ml. of this mixture being added to the uptake medium. The uptake of both I-131 sodium L-thyroxine and I-131 sodium triiodothyronine increases rapidly with previous incubation of the plasma with stable thyroxine. The L-triiodothyronine reached a plateau more rapidly (5 or 10 μg. per ml.) and at a higher level (53 per cent uptake) than the L-thyroxine (30 to 35 μg. per ml. and 30 per cent uptake).

The sodium fluoride treated cells and the 21-day-old refrigerated cells demonstrate the same uptake for the two compounds as do normal cells

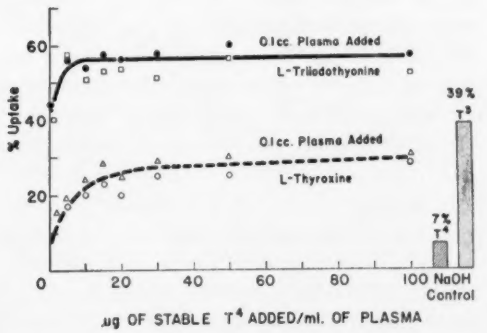

FIG. 3. THE EFFECT ON THE UPTAKE OF RADIOACTIVE I-131 L-THYROXINE AND L-TRIIODOTHYRONINE BY HUMAN WASHED RED CELLS PRODUCED BY THE ADDITION OF FRESH HUMAN PLASMA PREVIOUSLY INCUBATED WITH STABLE L-THYROXINE

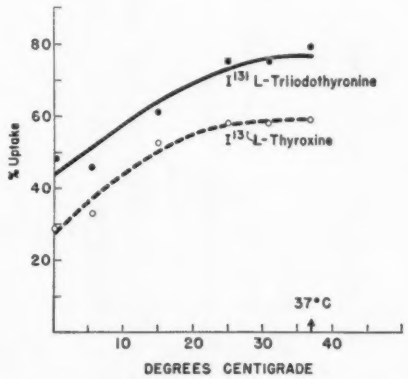

FIG. 4. THE EFFECT OF COLD ON THE UPTAKE OF RADIOACTIVE I-131 L-THYROXINE AND L-TRIIODOTHYRONINE BY WASHED HUMAN RED CELLS

provided fresh matched plasma is used with the old cells. Cold inhibits the uptake of both compounds by the cells. This decreases sharply as the temperature goes below 20°C (Figure 4).

Human red cell stroma exhibits much the same uptake and recovery characteristics that the intact cells do. Washed stroma takes up an average of 72 per cent (range 70 to 73 per cent) of the L-thyroxine in the medium and 76 per cent (range 75 to 78 per cent) of the L-triiodothyronine. NaI uptake is only 10 per cent. The addition of a small amount of plasma to the medium reduces the uptake greatly, 0.50 ml. reducing it to 15 per cent in the case of L-thyroxine. Incubation with fresh plasma recovers all but 13 per cent (range 11 to 14 per cent) of the L-thyroxine and 25 per cent (range 24 to 28 per cent) of the L-triiodothyronine originally associated with the stroma. Again saline alone is ineffective. The uptake and recovery for the stroma is presented in Figure 5.

DISCUSSION

Deiss, Albright, and Larson (1–3) have demonstrated the binding properties of serum proteins for L-thyroxine and L-triiodothyronine by paper radioelectrophoresis. They have shown by both *in vivo* and *in vitro* experiments that both L-thyroxine and L-triiodothyronine associate with a serum protein just ahead of the alpha-2 globulin zone which has been termed thyroxine-binding-globulin or TBG. L-thyroxine is also capable of

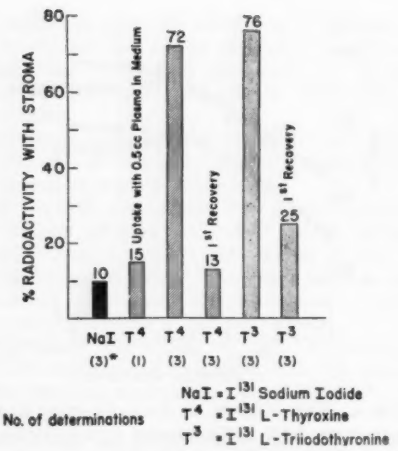

FIG. 5. THE UPTAKE OF RADIOACTIVE I-131 L-THYROXINE AND L-TRIIODOTHYRONINE BY RED CELL STROMA, THE EFFECT OF FRESH HUMAN PLASMA ON THE UPTAKE OF L-THYROXINE, AND THE AMOUNT OF RADIOACTIVITY REMAINING WITH THE STROMA AFTER THE RECOVERY OF THE RADIOACTIVE MATERIAL BY FRESH HUMAN PLASMA

displacing triiodothyronine from this protein indicating that the protein has a greater affinity for the tetraiodinated compound. This has also been demonstrated by Robbins and Rall (7). Furthermore, when TBG has previously been saturated with hormone, triiodothyronine trails off over the electrophoretic pattern with no quantitative relationship to protein indicating that it is not specifically bound by other serum proteins, while L-thyroxine is shown to associate with albumin. These workers feel this may account for a greater diffusibility of triiodothyronine and hence its more rapid disappearance from the serum.

Previous studies from our laboratory have shown that human red cells also have a binding or assimilating capacity for L-thyroxine and L-triiodothyronine (4). In this case the affinity of the intact red cells seemed to be greater for the triiodinated compound. Human plasma reduces the uptake of both compounds greatly; however, it is more effective in reducing the uptake of L-thyroxine than it is of L-triiodothyronine. This may also explain the apparent greater uptake of the L-triiodothyronine. Hamolsky (8) has presented evidence that the "uptake" by human red cells of L-triiodothyronine from plasma was greater than that of L-thyroxine. He also demonstrated that there was a greater "uptake" of L-triiodothyronine by human red cells from hyperthyroid plasma than from euthyroid plasma (8). In the present study the fact that fluoride ion or 21 days of cold storage have no effect on the cell uptake is evidence against this uptake being directly dependent on cell respiration although the red cell is considerably more inactive metabolically than other body cells. Christensen, Riggs, and Ray (9) have found difficulty in inhibiting the "uptake" of amino acids by red cells with enzyme poisons. Further support, however, that in the case of the iodinated amino acids, L-thyroxine and L-triiodothyronine, the red cell uptake is not directly dependent on respiration is provided by the fact that red cell stroma in which all respiratory and enzymatic processes are destroyed demonstrates much the same phenomenon.

The fact that fresh plasma mixtures are successful in recovering both the labeled hormones from the cells and the stroma is further evidence that this uptake is an equilibrium phenomenon between receptors for the hormones on the plasma proteins and receptors on the red cell, perhaps on the "red cell fixed framework" or stroma which is thought to consist of a complex structure of protein and lipid molecules. Cold inhibition of the uptake is compatible with an equilibrium phenomenon.

Evidence has been presented by Hoch and Lipmann (10) in experiments with liver mitochondria (rat and hampster) which indicates a binding capacity of cellular structures for thyroxine and triiodothyronine which is not immediately related to its metabolic activity. They have shown that while 80 to 90 per cent of the added hormone in a medium surrounding the mitochondria will attach itself to the mitochondria, there was no correlation between this absorption and the metabolic effect of the hormones as they measured it.

In the studies reported here the smaller cellular uptake of L-thyroxine and the more efficient recovery of it by plasma as compared to L-triiodothyronine is perhaps explained on the more avid binding by TBG and albumin of the thyroxine. One will note that the uptake by the stroma of the two compounds is very similar. The recovery of radioactivity from the stroma by plasma was greater for thyroxine (Figure 5). The washed

cells may still have a small component of plasma with them which would explain the decreased uptake of L-thyroxine as compared to uptake by stroma.

We are aware of the fact that we are assuming that the radioactivity recovered from the tagged red cells by the fresh plasma is still attached to the original label, the thyroid hormones. It is not in the form of inorganic iodide or it would have been washed away from the cell by the saline washings. Studies are now in progress to show that the recovered radioactivity is still attached to the original label.

Further evidence for the role played in the uptake by the binding capacities of plasma for the two hormones is demonstrated when the plasma added to the medium is previously incubated with stable thyroxine. The increase in uptake by the cell of both labeled L-thyroxine and L-triiodothyronine when the plasma has been previously incubated with small amounts of stable thyroxine may possibly be explained on the basis of saturation of receptors on the plasma proteins by the stable thyroxine. The fact that triiodothyronine reaches its maximum uptake much more rapidly than thyroxine can be explained on the basis of it being necessary to bind fewer plasma receptors (namely TBG alone) before triiodothyronine becomes fairly "unbound" to plasma protein. In the case of tetraiodothyronine (thyroxine) it may be necessary to saturate some albumin receptors also.

The presence of human fresh plasma inhibits the uptake by the intact human red cell of radioactive L-thyroxine to a greater degree than it does radioactive L-triiodothyronine (4). This could mean a greater binding capacity of the plasma for L-thyroxine or a greater binding capacity or uptake capacity by the intact red cell for triiodothyronine. The fact that the uptake or binding by the human red cell stroma is approximately equal (Figure 5) suggests that the above results are due to a greater binding capacity by human plasma for L-thyroxine. This is further substantiated by the fact that it is possible to remove approximately twice as much L-thyroxine as L-triiodothyronine from human red cell stroma using human fresh plasma (Figure 5).

As stated previously Hoch and Lipmann (10) presented data showing that there was no correlation between metabolic effects and the amount of hormone absorbed by the mitochondria. They also emphasize that activity of the hormone depends upon ability to permeate the cell wall, and suggest that triiodothyronine may be more active because of greater permeability of the cell wall. A second factor might be that the hormone is unavailable due to binding by plasma protein. Our studies suggest that thyroxine would be less available because of the strong binding properties of plasma for this tetra-iodinated compound compared to the tri-iodinated compound.

It should be emphasized that these *in vitro* studies with human erythrocytes and thyroid hormones may have no *in vivo* counterpart. The amount of thyroxine found in the red cell from *in vivo* studies appears to be negligible (11). Studies are in progress using other types of cells to study the binding phenomenon of thyroid hormones.

CONCLUSIONS

1. The uptake or binding by the human red cell of radioactive I-131 labeled L-thyroxine and L-triiodothyronine is not inhibited by the fluoride ion or storage of the cells for twenty-one days but is inhibited by cold.

2. Human red cell stroma was also shown to take up or bind radioactive I-131 labeled L-thyroxine and L-triiodothyronine.

3. The uptake or binding of the thyroid hormones by human erythrocytes, human erythrocyte stroma, and human plasma appears to be a physical chemical phenomenon not directly dependent on enzymatic processes of red cell respiration.

4. The greater binding capacity of human plasma for L-thyroxine as compared to L-triiodothyronine was again demonstrated.

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THE EFFECT OF ACUTE ELEVATION OF THE PLASMA CHLORIDE CONCENTRATION ON THE RENAL EXCRETION OF BICARBONATE DURING ACUTE RESPIRATORY ACIDOSIS [1]

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It has been postulated recently that renal bicarbonate reabsorption is *entirely* dependent on the exchange of hydrogen ions, derived from the dissociation of carbonic acid within the cells of the renal tubule, for the fixed base of filtered bicarbonate in the tubular urine (1–3). According to this theory, an elevation of the CO_2 tension of the extracellular fluid results in an increase in the rate of bicarbonate reabsorption because it leads to an increase in the rate of hydrogen ion secretion.

Earlier studies, however, indicate that chloride reabsorption may interfere with bicarbonate reabsorption presumably because of competition between these two ions for some common transport mechanism in the proximal tubule (4). Since plasma pCO_2 was apparently within normal range in those experiments, it was thought of interest to study the effect of hyperchloremia on bicarbonate reabsorption in the presence of an elevated extracellular pCO_2.

Our results indicate that under these circumstances the rate of bicarbonate reabsorption does not depend entirely on the CO_2 tension, but may, in part, depend upon the rate of chloride ion reabsorption.

METHODS

Normal adult mongrel female dogs were anesthetized by the intravenous administration of 0.46 to 0.62 mg. of sodium pentobarbital per kilogram of body weight. The trachea was cannulated with a "Y"-shaped glass tube, the two free arms of which served as inspiratory and expiratory airways by means of flutter valves. The animals breathed spontaneously throughout the entire experiment. The abdomen was opened by a midline incision and each ureter cannulated with a polyethylene tube. The abdomen was then closed by a continuous silk suture and the polyethylene tubes brought through the suture line, care being taken not to kink the ureters and tubes. Urine was collected directly under mineral oil contained in a graduated cylinder.

During the successive periods of study, the dog breathed first room air, then a mixture of CO_2 and air containing 10 per cent of CO_2.

The different solutions were administered at desired rates by means of a constant-speed Bowman pump via a cannulated brachial vein. As the operative procedure was begun, the animal was given intravenously within a 5 to 24-minute period 400 ml. of a 0.15 molar sodium bicarbonate solution containing 0.375 Gram per cent of creatinine. Then a sustaining infusion containing from 0.93 Gram per cent of $NaHCO_3$ to 1.175 Gram per cent of $NaHCO_3$ (0.11 to 0.14 molar solution) was administered at rates varying between 6.4 and 18.5 ml. per minute. This infusion also contained an amount of creatinine such that the dog received 15 mgm. of creatinine per minute. When the operative procedures were completed, a fifteen to thirty-minute equilibration period was allowed. Then control determinations were made, the animal breathing room air and receiving the sustaining infusion. The dog then inhaled the air-CO_2 mixture and after thirty minutes of administration of this mixture, a second set of determinations was made, the dog still receiving the same sustaining infusion as during the first set of determinations. Chloride loading was then accomplished by the rapid administration of a priming injection of 30 to 45 ml. of a 3.33 Gram per cent of sodium chloride solution. The sustaining infusion was then changed to one containing 1.68 Gram per cent to 2.3 Gram per cent of $NaHCO_3$ (0.2 M to 0.27 M) and 4.35 Gram per cent to 7.6 Gram per cent of NaCl. It was delivered at rates of from 3.2 to 6.4 ml. per minute. After thirty minutes of administration of this infusion, determinations were again made. In some experiments the first control period (room air breathing) was omitted. The exogenous creatinine clearance was taken as equal to the glomerular filtration rate (GFR).

Arterial blood samples were obtained at the midpoint of each clearance period from a needle inserted into a femoral artery. The blood was collected anaerobically and rendered incoagulable with heparin. The pH of the whole blood was determined immediately on a 0.5-ml.

[1] Supported by grants-in-aid from the New York Heart Association and the American Heart Association.

[2] Fellow of the New York Heart Association.

[3] Frances H. Zabriskie Fellow, St. Luke's Hospital.

TABLE I

Effect of sodium chloride infusion on the excretion of chloride and bicarbonate during acute respiratory acidosis in the anesthetized dog

Expt. No.	Period	Urine flow ml./min.	GFR ml./min.	Plasma				Urine			Bicarbonate					Chloride	
				Cl^- mEq./L.	HCO_3^- mEq./L.	pH	pCO_2 mm. Hg	Cl^- mEq./L.	HCO_3^- mEq./L.	pH	Filtered μEq./min.	Excreted μEq./min.	Reabsorbed μEq./min.	Reabsorbed mEq. per L. of filtrate	Reabsorbate mEq./L.	Reabsorbed mEq. per L. of filtrate	Reabsorbate mEq./L.
1	Air	2.0	52.8	102.8	34.2	7.53	43	7.0	183.6	7.90	1,897	367	1,530	29.0	30.1	107.7	111.9
	CO_2	2.7	55.8	95.9	41.3	7.27	93	5.0	140.8	7.75	2,422	373	2,049	36.8	38.6	100.5	105.5
	CO_2+5% NaCl	9.4	50.3	127.0	40.1	7.25	95	98.0	68.0	7.41	2,119	636	1,483	29.5	36.2	115.2	141.7
2	Air	2.08	36.3	98.0	39.3	7.46	57	3.0	155.8	7.77	1,499	323	1,177	32.5	34.4	103.0	108.9
	CO_2	2.33	38.7	92.4	45.0	7.25	106	1.3	115.7	7.59	1,824	269	1,555	40.6	42.8	97.0	103.0
	CO_2+5% NaCl	9.47	37.3	127.3	44.7	7.20	120	104.0	63.0	7.25	1,756	597	1,150	30.9	41.4	107.0	143.7
3	Air	4.81	61.6	101.2	35.0	7.49	48	24.8	85.9	7.70	2,259	410	1,849	30.0	32.7	105.0	113.6
	CO_2	5.01	58.1	95.0	43.4	7.26	101	11.4	67.2	7.47	2,647	337	2,310	39.8	43.5	99.0	107.9
	CO_2+5% NaCl	15.4	52.4	143.0	44.6	7.25	117	113.6	46.9	7.19	2,450	722	1,728	33.0	46.7	117.0	165.5
4	Air	4.0	76.8	102.3	32.3	7.46	47	57.3	124.1	7.81	2,601	480	2,121	27.5	29.1	104.3	110.1
	CO_2	3.1	64.3	97.6	38.8	7.27	88	33.3	108.9	7.66	2,620	332	2,288	35.6	37.4	101.0	106.0
	CO_2+5% NaCl	16.2	63.8	132.7	39.7	7.24	96	117.3	49.3	7.30	2,655	795	1,861	29.2	39.1	109.9	149.5
5	CO_2	2.3	85.7	105.0	34.5	7.24	84	37.5	165.9	7.70	3,097	377	2,720	31.8	32.6	108.8	111.7
	CO_2+4.35% NaCl	11.5	102.4	122.9	34.8	7.21	90	131.5	91.6	7.49	3,741	1,049	2,697	26.4	29.7	114.2	128.7
	CO_2+4% NaCl +0.5% KCl	12.5	104.4	141.3	33.7	7.18	92	141.0	93.7	7.48	3,695	1,172	2,519	24.2	27.5	131.5	149.4
6	CO_2	9.0	47.9	89.0	48.9	7.41	80	1.6	74.2	7.64	2,458	668	1,790	37.4	46.1	93.2	114.8
	CO_2+5% NaCl	10.9	46.7	115.2	46.1	7.37	83	38.5	75.7	7.71	2,258	825	1,433	30.8	40.2	112.0	146.4
7	CO_2	3.6	36.2	81.9	54.5	7.38	97	0.9	115.2	7.65	2,068	413	1,652	45.8	50.9	85.9	95.4
	CO_2+5% NaCl	7.3	37.4	109.8	52.2	7.32	105	62.4	91.0	7.50	2,047	662	1,386	37.1	46.1	103.2	128.3
8	CO_2	5.66	53.6	87.0	46.3	7.32	94	3.0	99.6	7.60	2,602	564	1,988	38.1	42.6	91.1	98.7
	CO_2+5% NaCl	6.76	52.6	119.5	40.9	7.29	89	72.5	85.2	7.52	2,257	575	1,682	31.9	36.7	116.1	133.6
9	CO_2	4.44	47.0	85.8	48.4	7.43	75	8.9	148.6	7.67	2,385	660	1,725	36.7	40.5	89.1	98.5
	CO_2+7.6% NaCl	10.5	52.6	110.3	44.4	7.35	84	88.6	79.9	7.48	2,451	838	1,613	30.7	38.3	96.9	121.0
10	Air	6.56	46.1	88.4	47.2	7.61	49	13.5	150.2	7.83	2,287	984	1,303	28.3	33.0	90.9	106.1
	CO_2	7.67	43.1	80.5	57.7	7.42	92	5.5	124.2	7.69	2,609	951	1,658	38.6	46.9	83.5	102.5
	CO_2+5% NaCl	11.0	48.5	110.1	51.7	7.37	92	71.5	109.2	7.59	2,636	1,202	1,434	29.6	38.3	99.4	128.6

portion of the sample and the rest of the sample was delivered under mineral oil into a centrifuge tube and centrifuged. The plasma was then immediately analyzed for CO_2 content.

Creatinine was determined in diluted urine and protein free filtrates of plasma by the method of Kennedy, Hilton, and Berliner (5). Total CO_2 in plasma and urine was determined by the manometric extraction technique of Van Slyke and Neill (6). The pH of the whole blood and urine was determined anaerobically at room temperature with a Beckman model G pH meter equipped with a syringe-type glass electrode. A correction factor (7) of 0.014 pH units per degree of difference between room and body temperatures was used in the calculation of the pH. Concentrations of bicarbonate and dissolved CO_2 were calculated from the Henderson-Hasselbalch equation employing a pK′ of 6.1 for carbonic acid, and an α equal to 0.0301 for plasma and 0.0309 for urine. The concentrations of sodium and potassium in plasma and urine were determined with an internal standard flame photometer. Chloride concentration in the plasma was determined on protein-free filtrates of plasma according to the method of Schales and Schales (8). A modified Volhard titration method (9) was used in determining the chloride concentrations of urine. The amount of each ion filtered per minute was calculated by multiplying its plasma concentration by the glomerular filtration rate. A Donnan distribution factor of 0.95 for Na^+ and K^+ and 1.05 for Cl^- and HCO_3^- was employed in this calculation. The rate of excretion of each ion was calculated by multiplying its urine concentration by the urine flow. The rate of reabsorption of water or any ion was taken as the difference between its rate of filtration and its rate of excretion. To facilitate the analysis and the discussion of the results, the amount of a given ion reabsorbed per liter of glomerular filtrate was also calculated by dividing the rate of reabsorption of that ion expressed in mEq. per min. by the filtration rate expressed in ml. per min. The concentration of each ion in the volume of water reabsorbed or "reabsorbate" was calculated by dividing the rate of reabsorption of each ion (mEq. per min.) by the rate of water reabsorption (ml. per min.), the resultant concentration being expressed in terms of mEq. per L.

RESULTS

Ten experiments were performed on ten animals. The results are summarized in Table I and the protocol of one of these experiments is given in Table II. In four experiments (Nos. 1, 2, 3, and 4) the animals were studied, first, while breathing room air and receiving intravenously an infusion containing a 0.11 molar solution of sodium bicarbonate delivered at a rate of 6.4 ml. per minute, then while breathing the air-CO_2 mixture and receiving the same infusion, and finally while continuing to breath the same air-CO_2 mixture but receiving intravenously a 0.2 molar solution of $NaHCO_3$ containing 5 Gram per cent of NaCl. In one experiment (No. 5) the animal was studied initially while breathing the air-CO_2 mixture and receiving intravenously a 0.11 molar solution of

TABLE II

Experiment illustrating the effect of sodium chloride infusion on the excretion and reabsorption of bicarbonate during acute respiratory acidosis

Time	GFR	Urine flow	Reabsorbate volume	Plasma			Urine			Bicarbonate*				
					HCO_3^-	pCO_2		HCO_3^-	pCO_2	Filtered	Excreted	Reabsorbed	Reabsorbed per L. filt.	Conc. in reabsorbate
min.	*ml./min.*	*ml./min.*	*ml./min.*	pH	*mEq./L.*	*mm. Hg*	pH	*mEq./L.*	*mm. Hg*	*μEq./min.*	*μEq./min.*	*μEq./min.*	*mEq.*	*mEq./L.*
				Dog 10, ♀, 15.9 Kg., breathing room air										
0–8	prime: 400 ml. 0.375% Creatinine in 1.25% $NaHCO_3$													
8	Begin infusion: 0.081% Creatinine in 1.175% $NaHCO_3$ at 18.5 ml. per min.													
77–81	46.8	6.25	40.5	7.61	47.2	49	7.84	154.2	91	2,321	964	1,357	29.0	33.5
82–86	45.4	6.87	38.5	7.61	47.2	49	7.82	146.2	90	2,252	1,004	1,248	27.5	32.4
86	Begin inhale 10% CO_2 and 90% air for duration of experiment													
116–119	44.0	8.34	35.7	7.42	57.7	92	7.68	120.7	103	2,666	1,007	1,659	37.7	46.5
120–123	42.1	7.0	35.1	7.42	57.7	92	7.69	127.7	106	2,551	894	1,657	39.4	47.2
123.5	prime: 45 ml. of 3.33% NaCl													
124	Change infusion to: 0.278% Creatinine and 2.3% $NaHCO_3$ in 5% NaCl at 5.4 ml. per min.													
154–157	48.3	11.18	37.1	7.37	51.7	92	7.59	110.2	115	2,627	1,232	1,395	28.9	37.6
158–161	48.7	10.82	37.9	7.37	51.7	92	7.58	108.2	116	2,644	1,171	1,473	30.2	38.9

* See methods section for calculations of derived data.

$NaHCO_3$ administered at the rate of 6.4 ml. per minute. The chloride loading was then accomplished first by the administration of a 0.2 molar solution of $NaHCO_3$ containing 4.35 Gram per cent of NaCl at the same rate, then by changing the infusion to a 0.2 molar solution of $NaHCO_3$ containing 4.0 Gram per cent of NaCl and 0.5 Gram per cent of KCl delivered at the same rate of 6.4 ml. per min. Because the urine flow always rose sharply in these first five experiments when the hypertonic chloride infusion was given, an attempt was made to prevent this in the last five experiments (Nos. 6, 7, 8, 9, and 10) by administering a 0.11 to 0.14 molar solution of $NaHCO_3$ at higher rates, *i.e.*, 11 to 18.5 ml. per min. The chloride loading was then achieved by administering a 0.2 to 0.27 molar solution of $NaHCO_3$ containing 5 to 7.6 Gram per cent of NaCl at slower rates of 3.2 or 5.4 ml. per min. In four of these last five experiments (Nos. 6, 7, 8, and 9) the initial period of breathing room air was omitted.

In all experiments, during chloride loading, the amount of chloride filtered and the amount of chloride excreted in the urine both increased. Concomitantly, moreover, the rate of chloride reabsorption increased, as well as the concentration of chloride in the reabsorbate and the amount of chloride reabsorbed per liter of glomerular filtrate.

In all experiments, the load of filtered bicarbonate remained essentially unchanged during chloride loading, rising slightly in four experiments (Nos. 4, 5, 9, and 10) and decreasing slightly in six experiments (Nos. 1, 2, 3, 6, 7, and 8). In all experiments, however, whether the filtered bicarbonate slightly rose or decreased, the amount of HCO_3^- excreted in the urine increased during chloride loading and the amount of HCO_3^- reabsorbed per liter of glomerular filtrate decreased.

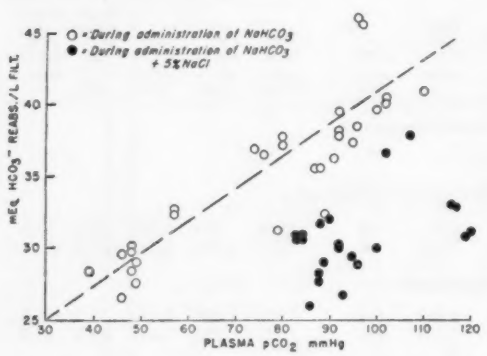

FIG. 1. RELATIONSHIP BETWEEN PLASMA CO_2 TENSION AND AMOUNT OF BICARBONATE REABSORBED PER LITER OF GLOMERULAR FILTRATE IN ACUTE RESPIRATORY ACIDOSIS BEFORE AND DURING INFUSION OF SODIUM CHLORIDE

As can be seen in Table I, bicarbonate reabsorption increased as the plasma pCO_2 rose, which confirms the work of other investigators (2, 3, 10). During chloride administration, however, there occurred a fall in the rate of bicarbonate reabsorption and a rise in the bicarbonate excretion, despite the persistence in the elevation of the plasma CO_2 tension. Figure 1 expresses graphically the relationship between the plasma pCO_2 and the amount of bicarbonate reabsorbed per liter of glomerular filtrate during the administration of the air-CO_2 mixture and the infusion of bicarbonate, both before and during chloride loading. The results observed before chloride loading are similar qualitatively and quantitatively to those reported by Brazeau and Gilman (2) as well as Dorman, Sullivan, and Pitts (3). The points representing such clearance periods are closely grouped along a straight line. However, the points representing the relationship between plasma pCO_2 and the amount of HCO_3^- reabsorbed per liter of glomerular filtrate during chloride loading are aberrant in their location from the first group of points. Indeed, during chloride administration, even at CO_2 tensions ranging between 90 and 100 mm. Hg, bicarbonate reabsorption falls to between 25 and 30 mEq. per liter of glomerular filtrate, values only found at CO_2 tensions of 35 to 50 mm. Hg before the chloride administration.

Plasma potassium levels were consistently between 1.8 and 2.8 mEq. per L. throughout all experiments with the exception of the final period of experiment No. 5 when the dog received KCl incorporated into the chloride infusion. Urine potassium excretion, with this latter period excepted, ranged between 35 and 104 μEq. per min. Both blood and urine values remained very steady throughout each experiment and potassium excretion did not appear to be influenced either by elevation of CO_2 tensions or by the combination of elevated pCO_2 and chloride loading.

DISCUSSION

The data presented above indicate that during acute respiratory acidosis, the renal excretion of bicarbonate was significantly increased by acute elevation of the plasma chloride concentration. Since the filtered load of bicarbonate remained essentially unchanged following the chloride infusions, the increased rate of bicarbonate excretion was entirely due to a reduction in the renal tubular reabsorption of bicarbonate. This occurred despite a tendency for extracellular CO_2 tension to increase during the chloride loading. There seem to be several theoretically possible explanations for this increase in HCO_3^- excretion: 1) Chloride loading interfered with H^+ secretion by the renal tubular cells, less HCO_3^- was converted to CO_2 and H_2O, more became available in the tubular fluid and was excreted as $NaHCO_3$; 2) Chloride loading did not interfere either actively or directly with the reabsorption of HCO_3^- but promoted an osmotic diuresis which caused more of the filtered HCO_3^- to be excreted; 3) The increase in chloride filtration and the subsequent increase in Cl^- reabsorption interfered directly with HCO_3^- reabsorption; or 4) HCO_3^- was actively secreted by the tubular cells.

It is unlikely that H^+ secretion was depressed by chloride loading in view of the fact that urine pH decreased and urine pCO_2 increased in most experiments following the chloride loading. The fall in urine pH *per se*, in alkaline urines such as these, could be explained solely by the fall in HCO_3^- concentration due to dilution (*i.e.*, osmotic diuresis) since the tubules are freely permeable to CO_2. However, the rise in urine CO_2 tension to values greater than that of the arterial plasma in the face of rising urine flows can best be explained on the basis of hydrogen ion addition to these urines resulting in titration of HCO_3^- to form additional carbonic acid. Such H^+ addition could come from distal tubular secretion of H^+ into a urine containing increasing amounts of HCO_3^-, or it could come from an increase in the excretion of acid buffers into this urine. Total buffer concentration was not measured in these experiments; but the concentration of one buffer (*i.e.*, creatinine) was measured and found to decrease. It does not seem unreasonable, therefore, to presume that the concentration of other buffers would likewise decrease as urine flow increased and hence the rise in urine pCO_2 appears to be the result of continued secretion of hydrogen ion into a highly alkaline urine. The decrease in HCO_3^- reabsorption following the chloride loading in the face of continued H^+ secretion must therefore be explained on the basis of some other mechanism.

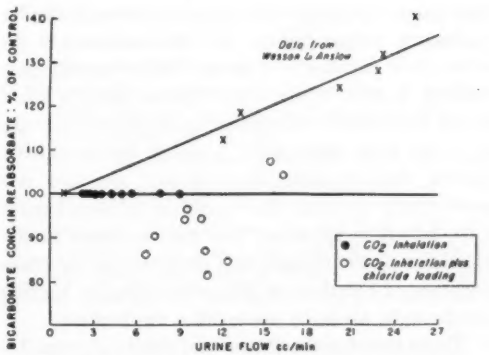

FIG. 2. PER CENT CHANGE IN BICARBONATE CONCENTRATION OF REABSORBATE PLOTTED AGAINST URINE FLOW

The points on the 100 per cent line represent control values observed during CO_2 inhalation. The open circles are the values obtained following the chloride loading, during continued CO_2 inhalation. The data from Wesson and Anslow (12) was obtained during mannitol diuresis under conditions of normal CO_2 tension.

It is difficult to hold that osmotic diuresis was responsible for the decreased reabsorption of bicarbonate because in 8 of 10 experiments there was a fall in the concentration of bicarbonate in the reabsorbate following the chloride loading. This occurred despite marked increases in urine flow and decreases in reabsorbate volume in four of these experiments (Nos. 1, 2, 5, and 9). If it is allowed that there is normally no limitation to the concentration of HCO_3^- in the reabsorbate one would expect that under a constant rate of HCO_3^- reabsorption (11) there would be an increase in HCO_3^- concentration of the reabsorbate as the urine volume increases and the volume of the reabsorbate falls. This has been shown by Wesson and Anslow (12) to occur during mannitol diuresis. In Figure 2 is plotted the per cent change in the bicarbonate concentration of the reabsorbate following chloride loading compared with the data of Wesson and Anslow (12) obtained under conditions of mannitol loading. During mannitol diu-

resis there is a progressive linear increase in the bicarbonate concentration of the reabsorbate as urine flow increases, whereas following chloride loading in most of our experiments there is a fall in the bicarbonate concentration of the reabsorbate as urine flow increases. Since it has also been shown that osmotic diuresis *per se* does not particularly increase the excretion of bicarbonate (13, 14) it is apparent that the increases in the excretion of bicarbonate and decreases in the reabsorption of bicarbonate following chloride loading are brought about by some other mechanism.

There remains the theoretical possibility that bicarbonate may have been secreted by the tubular cells and that such a secretion of HCO_3^- by the tubular cells may have been increased by chloride loading, thus accounting for the observed increase in excretion and apparent decrease in reabsorption of bicarbonate during chloride loading. It can only be stated in this regard that there is no direct experimental evidence that such a mechanism exists.

It appears then that during acute respiratory acidosis an increase in the amount of chloride reabsorbed interferes with the reabsorption of bicarbonate. This is in accord with the earlier observations made by Pitts and Lotspeich (4) on animals with presumably normal CO_2 tensions and is entirely consonant with the theory that there is competition between these two ions for a common reabsorptive transport mechanism. Indeed, it was originally suggested by these investigators that a dual process of bicarbonate reabsorption existed whereby most of the bicarbonate filtered at the glomerulus was reabsorbed by a specific anion transport in the proximal tubule. In this schema some of the bicarbonate ions not reabsorbed by the proximal tubule were reabsorbed in the distal tubule by a process involving exchange of H^+ ions originating within the tubular cells for Na^+ ions derived from the tubular urine.

It was later observed by Berliner (1) that following the administration of Diamox®, the amount of the additional bicarbonate excreted in the urine was too great to be accounted for solely by the abolition of the distal hydrogen ion secretory mechanism. Berliner therefore proposed that bicarbonate reabsorption throughout the *entire* renal tubule was dependent upon the hydrogen ion secretion mechanism. This concept was supported by the recent studies of Brazeau and Gilman (2) and Dorman, Sullivan, and Pitts (3). Working independently, these investigators noted that as the CO_2 tension of the extracellular fluid is raised, there is a concomitant linear increase in renal bicarbonate reabsorption. Since, presumably, H^+ secretion by the renal tubules increases with increasing pCO_2, they concluded that the increased rate of HCO_3^- reabsorption could best be explained by the assumption that the reabsorptive mechanism was entirely dependent upon the exchange of H^+ ions derived from carbonic acid in the tubular cells for fixed base of filtered bicarbonate in the tubular urine.

This $H^+ - B^+$ exchange theory of the renal regulation of bicarbonate excretion however, does not adequately account for the reciprocal changes in the plasma HCO_3^- levels which accompany rises and falls in plasma Cl^- levels. Indeed, it was this phenomenon, together with the observation that increased Cl^- loading facilitated HCO_3^- excretion, which led Pitts and Lotspeich (4) to postulate specific ionic reabsorption of bicarbonate by the proximal tubule. Moreover, although the theory adequately explains the renal compensations in acute and chronic respiratory acidosis and alkalosis, it does not satisfactorily explain the renal compensations for metabolic acidosis and alkalosis, as pointed out by Brazeau and Gilman (2) as well as by Relman, Etsten, and Schwartz (10). One wonders if the main weakness of the theory does not lie in the assumption that HCO_3^- reabsorption can only occur through H^+ ion secretion. If this were so, one would expect that the urine pH would decrease as HCO_3^- reabsorption increases. That this is not the case is apparent from analysis of the data published to date (1–3). In fact, in experiments on dogs with chronic respiratory acidosis (15), it can be seen that as HCO_3^- reabsorption increased with increasing plasma pH levels, the urine became more alkaline and the pCO_2 of the urine declined.

It would appear then, that bicarbonate reabsorption is not entirely due to $H^+ - B^+$ exchange since it is possible to elicit a reciprocal relationship between chloride and bicarbonate reabsorption and to induce an increase in the reabsorption of one of these ions and a decrease in the reabsorption of the other while the pCO_2 remains constant. Furthermore, this reciprocal relationship

was observed in the presence of two factors which have been demonstrated both to reduce chloride reabsorption and to increase bicarbonate reabsorption, *i.e.*, (a) high plasma CO_2 tensions (2, 3), and (b) a probable state of potassium depletion (16, 17) resulting from the infusion of large amounts of sodium bicarbonate, at least as judged by the low plasma potassium levels.

SUMMARY AND CONCLUSIONS

1. In acute experiments performed on anesthetized dogs, it has been observed that:

A. In acute respiratory acidosis, the amount of bicarbonate excreted in the urine decreases and the amount of bicarbonate reabsorbed increases, whereas the amount of chloride excreted decreases and the amount of chloride reabsorbed decreases;

B. The administration of chloride during respiratory acidosis results in an increase in the amount of bicarbonate excreted and a decrease in the amount of bicarbonate reabsorbed.

2. The phenomena observed suggest the possibility that at least part of the reabsorption of bicarbonate is ionic and not dependent upon hydrogen ion secretion by the tubule. This part of the bicarbonate reabsorption is affected by the reabsorption of chloride in such a manner as to suggest competition between the chloride and bicarbonate ions for a common reabsorptive transport mechanism in the tubule.

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PLACENTAL PASSAGE OF 17-HYDROXYCORTICOSTEROIDS: COMPARISON OF THE LEVELS IN MATERNAL AND FETAL PLASMA AND EFFECT OF ACTH AND HYDROCORTISONE ADMINISTRATION [1]

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A progressive rise in the levels of free plasma 17-hydroxycorticosteroids (17-OH-CS) during the course of pregnancy has been observed by a number of investigators (1–4). These findings are in conformity with the increase in urinary glucocorticoids previously reported (5). Normal adult concentrations have been found in the umbilical vein plasma obtained from the newborn infant at the time of delivery (2, 6–8), but significantly lower levels were observed in infants born by cesarean section (7).

The site of production and the nature of plasma 17-OH-CS found in increased amounts during pregnancy and at delivery has not yet been elucidated. The maternal and fetal adrenals, as well as the placenta could be the source of C_{21} steroids with an α-ketolic side chain. A rise in plasma and urinary corticosteroids has been reported in pregnant Addisonian patients (9, 10) which has suggested that during pregnancy an extra-adrenal source, namely the placenta, may be secreting such hormones. The prompt decrease in plasma 17-OH-CS in the newborn infant during the first few days of life (6), and the very low umbilical cord plasma concentrations in infants delivered by cesarean section (7) could be interpreted as indicating a lack of fetal production of corticosteroids. The rather constant ratio of 17-OH-CS in the maternal as compared to the umbilical cord plasma in cases of vaginal delivery has suggested that fetal levels were only the reflection of maternal concentrations (8).

The present study was designed to clarify the role of placental transmission of free 17-OH-CS and to further identify the corticosteroids found in maternal and fetal circulations.

METHODS

The subjects in the present study were 33 pregnant females, 15 to 40 years of age. Vaginal delivery was performed in nine cases (Case Numbers 31 to 39). Cesarean section was deemed advisable in five cases because of placenta previa (Case Numbers 42 and 44) or uterine inertia (Case Numbers 40, 41, 43). Nineteen patients had an "elective, repeat cesarean" (Case Numbers 45 to 63), the section being performed approximately at term and before onset of labor. The majority of the vaginal deliveries were conducted under systemic analgesia and pudendal block anesthesia. Cesarean sections were usually accomplished with local infiltration of novocaine and intravenous Pentothal® anesthesia. None of the subjects had toxemia or complications other than mechanical ones necessitating a cesarean section.

Blood samples were drawn in a heparinized syringe and the red cells were immediately separated from the plasma. The concentration of free 17-OH-CS was determined by the method of Nelson and Samuels (11).

(A) In the vaginal series, blood was collected during labor, 30 minutes to 4 hours prior to delivery. In the cesarean group (Case Numbers 40–51) specimens were obtained 30 minutes preceding anesthesia. A second sample was drawn simultaneously from the mothers and infants at the time of delivery. Finally, maternal blood was taken between 8 and 9 A.M., on the third and sometimes seventh postpartum day.

(B) An intravenous infusion of 25 mg. of ACTH (Armour ACTHAR, lot no. J21812) [3] dissolved in 250 ml. of 5 per cent dextrose in water, was administered over a period of six hours to seven patients scheduled for an "elective, repeat cesarean section" (Case Number 52 to 58). The infusion was started at 8 A.M., after a

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[3] The ACTH preparation used in this study was generously provided by Armour Laboratories, Chicago, Illinois.

TABLE I

Levels of 17-OH-CS in maternal and cord plasma in cases of vaginal delivery and cesarean section

Delivery	Case Number	Age (yrs.)	Race	Duration of Pregnancy (Weeks)	17-OH-CS (μg. per 100 ml. plasma)				
					MATERNAL		CORD	MATERNAL Post Delivery	
					Pre Delivery	At Delivery		3rd Day	7th Day
Vaginal	31 D.B.	37	C	39	26.4	38.5	10.9	25.2	
	32 E.B.	36	C	37	24.0	41.5	18.6	14.9	
	33 P.M.	15	C	36	53.0	46.0	0.2	21.5	
	34 M.W.	22	C	39	47.5	52.0	27.0	43.0	
	35 F.S.	32	C	41	49.5	54.4	12.0	5.9	
	36 O.D.	35	C	41	41.8	56.0	16.5	16.5	
	37 M.M.	27	C	32	52.5	67.0	43.5	40.0	
	38 M.R.	28	W	37	71.0	76.1	65.8	30.5	
	39 J.R.	22	W	39	69.0	78.0	14.3	29.4	
Indicated Cesarean	40 S.M.	32	W	42	23.0	28.0	5.0	—	29.2
	41 S.W.	29	C	40	19.0	34.0	7.0	14.0	15.8
	42 H.M.	22	W	35	31.0	47.0	22.0	53.2	29.7
	43 M.P.	32	W	43	—	66.0	16.5	6.4	14.0
	44 I.G.	30	W	44	48.0	98.5	13.6	18.0	16.5
Elective Repeat Cesarean	45 E.McL.	27	C	19	6.8	8.6	0.6	—	—
	46 G.J.	25	W	38	14.0	20.6	4.3	25.0	20.3
	47 C.L.	34	W	38	13.6	21.5	1.4	25.5	10.5
	48 P.G.	26	W	39	17.0	23.0	5.0	16.0	13.2
	49 E.J.	20	C	38	10.2	32.4	9.5	—	—
	50 J.B.	26	W	39	18.4	33.5	8.6	18.4	19.4
	51 D.G.	28	C	38	24.4	35.8	0.3	—	—

control blood sample had been drawn, and terminated at the time of delivery, which took place at 2 P.M. Additional blood specimens were collected from the mother, two and four hours after the start of the infusion and simultaneously from the mother and the cord at delivery.

(C) An intravenous infusion of hydrocortisone (Upjohn, Cortef 0.5 per cent w/v) [4] dissolved in 250 ml. of 5 per cent dextrose in water was started at 8 A.M. and given over a period of 30 minutes to a second group of 5 patients (Case Numbers 59 to 63) scheduled for "elective, repeat cesarean section." The surgical procedure was begun just after the start of the infusion and delivery was performed at the termination of steroid administration. A control blood sample was obtained from the mother immediately prior to this procedure; maternal and cord specimens were collected simultaneously at delivery. Patients 59 to 61 received 50 mg. of hydrocortisone and patients 62 and 63, 100 mg.

(D) To characterize further the free plasma 17-OH-CS present in the maternal and fetal circulation at the time of delivery, blood samples were collected, pooled and extracted with chloroform. After evaporation the residue was chromatographed on Florisil columns and the 25 per cent methanol in chloroform eluate was evaporated. The residue was applied to Whatman No. 2 paper and chromatographed in the methanol: water (55:45)/benzene system of Bush (12). The chromatograms were studied as previously described (13).

[4] The I.V. preparation of Hydrocortisone was kindly supplied by the Upjohn Co., Kalamazoo, Michigan.

RESULTS

(A) Table I shows the levels of free 17-OH-CS in maternal plasma before and at delivery, and in the immediate postpartum period in pregnancies terminated by vaginal delivery, indicated cesarean section, and "elective, repeat cesarean section." These values are compared to the levels found in the umbilical vein plasma at birth. Labor generally caused a rise in the maternal concentrations as evidenced by the higher predelivery values in the vaginal series when compared to those obtained in the elective cesarean group. The trauma of parturition also appeared to increase the levels of the mother. Irrespective of the method of delivery, the 17-OH-CS concentration in the cord plasma was invariably less than that found in the mother (see Figures 1 and 2). Furthermore, the levels in the umbilical vein plasma of infants born by elective cesarean section were lower than those obtained in the other two groups of infants.

(B) The response of maternal 17-OH-CS levels to ACTH administration in cases of "elective repeat cesarean section" (see Table II) was similar to that observed in nonpregnant females (14). This is in contrast to the hyperresponsiveness reported by Christy, Wallace, and Jailer (15)

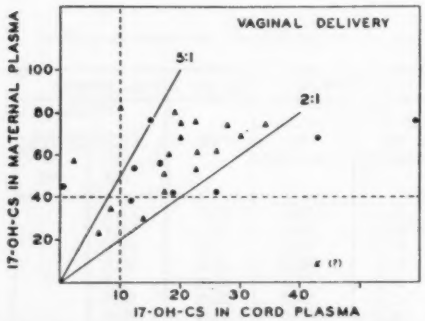

FIG. 1. RELATIONSHIP BETWEEN 17-OH-CS LEVELS IN MATERNAL AND CORD PLASMA IN CASES OF VAGINAL DELIVERY

The black circles are the values reported in the present paper; the black triangles are the values reported in a previous paper (8).

The horizontal broken line indicates the 40 μg. level for maternal plasma; most values are over this line. The vertical broken line indicates the 10 μg. level for cord plasma; most cord values are higher than 10 μg.

The full lines are indicative of a ratio maternal: cord value of 2:1 and 5:1. It can be seen that most values are situated between the two lines, demonstrating the existence of a concentration gradient between the maternal and cord 17-OH-CS.

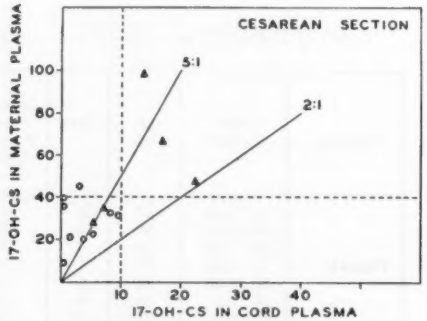

FIG. 2. RELATIONSHIP BETWEEN 17-OH-CS LEVELS IN MATERNAL AND CORD PLASMA IN CASES OF CESAREAN SECTION

The open triangles are the cases of "indicated cesarean"; the open circles, the "elective cesareans." The values of two cases reported in a previous paper (8) were added on the figure. It can be noted that in all the elective cesareans, the maternal values were lower than 40 μg. and those in cord blood lower than 10 μg.

in the third trimester of pregnancy; however, the method employed by these authors was less specific than the technique used in the present study and the former may be determining some steroids that the latter method does not. In blood specimens obtained simultaneously from the mother and the newborn infant at delivery, the concentration of 17-OH-CS in the cord plasma was one-third to one-half that found in the maternal plasma. These cord levels were without exception higher than those observed in the group delivered by "elective, repeat cesarean section," not stimulated with ACTH.

(C) The increase of the 17-OH-CS levels obtained in maternal and cord plasma was directly proportional to the amount of compound F administered (Table III). Although strikingly elevated, the levels in the cord plasma were invariably lower than the maternal values. The passage of hydrocortisone infused to the mother across the placenta to the fetus is demonstrated by comparing

TABLE II

Effect of ACTH administration on 17-OH-CS levels in maternal and cord plasma in cases of elective, repeat cesarean section

Case Number	Age (Yrs.)	Race	Duration of Pregnancy (Weeks)	Medication	17-OH-CS (μg. per 100 ml. plasma)				
					MATERNAL				CORD
					8 A.M. (Control)	10 A.M.	12 Noon	2 P.M. (Delivery)	2 P.M.
52 L.D.	27	W	38	25 units ACTH over 6 hours	30.4	50.5	71.5	61.0	30.5
53 B.C.	40	C	37	25 "	12.3	23.0	22.0	31.6	11.5
54 A.L.	39	C	39	25 "	15.7	30.8	36.0	34.0	15.0
55 B.K.	26	C	37	25 "	5.5	18.0	33.0	38.6	13.5
56 R.C.	37	C	42	25 "	14.7	23.0	20.0	36.2	13.7
57 C.D.	31	C	38	25 "	14.1	34.1	49.3	54.5	23.0
58 R.W.	24	C	38	25 "	22.5	41.0	44.7	48.1	21.7

TABLE III

Effect of compound F administration on 17-OH-CS levels in maternal and cord plasma cases of elective, repeat cesarean section

Case Number	Age (Yrs.)	Race	Duration of Pregnancy (Weeks)	Medication	17-OH-CS µg. per 100 ml. plasma		
					Maternal		Cord
					Control	On Cpd. F	
59 M.S.	39	W	39	50 mg.IV cpd.F	21.1	178.0	50.4
60 M.P.	31	W	38	50 mg.IV cpd.F	29.2	158.0	67.0
61 A.A.	20	W	39	50 mg.IV cpd.F	17.6	148.0	77.0
62 S.C.	23	C	39	100 mg.IV cpd.F	25.4	301.0	86.5
63 A.J.	36	C	38	100 mg.IV cpd.F	24.0	326.0	105.0

the 17-OH-CS levels in cord plasma in the control group of "elective repeat cesarean sections" and those in the group who received compound F.

(D) The method of Nelson and Samuels (11) was shown to measure mainly free plasma hydrocortisone. There exists a satisfactory correlation between results obtained by this method and paper chromatographic techniques in normal individuals and following ACTH administration (16), as well as in patients undergoing surgical or medical stress, and in moribund individuals (13). It was found in the present study that the values obtained for 17-OH-CS by the method of Nelson and Samuels were in good agreement with the amount of compound F eluted from the paper chromatogram of the corresponding extract (Table IV). A spot with the mobility of compound B could not be observed on the paper chromatograms of either maternal or fetal extracts. This possibly may be attributed to our use of methods not sufficiently sensitive to detect quantities of compound B less than 5 to 8 micrograms.

DISCUSSION

The role which adrenal secretion, tissue factors, alterations in the rate of hepatic degradation and conjugation, and kidney clearance, respectively, may play in contributing to the elevated levels of free plasma 17-OH-CS during pregnancy, labor and delivery is not known. That increased plasma concentrations do not necessarily reflect an increase in hormone production, is evidenced by the studies of corticosteroid metabolism in dying patients (17), and in patients subjected to surgical procedures (18, 19). In many instances, the maternal levels of 17-OH-CS in cases of vaginal delivery were found to be higher than those observed after administration of a maximal dose of ACTH to normal, nonpregnant females; this raises the

TABLE IV

Comparison of the results obtained by the method of Nelson and Samuels and paper chromatographic techniques

Sample*	Delivery	17-OH-CS µg./100 ml. (Nelson and Samuels)	Compound F µg./100 ml. (paper chromatography)
Maternal plasma	Vaginal delivery	56.0	47.6
Cord plasma	Vaginal delivery	21.0	14.5
Maternal plasma	Cesarean section	23.5	18.0

* Each sample was made up by pooling approximately equal amounts of plasma from three mothers or three umbilical cords.

possibility that extra adrenal factors might be involved, although it has been suggested that there is a hyperresponsiveness of the adrenal cortex to exogenous ACTH administration during the third trimester of pregnancy (15).

The infusion of compound F to the mother produced an increase in her 17-OH-CS levels as normally expected, but the fact that cord values were higher than those obtained when no steroid was administered strongly suggests that the placenta is permeable to compound F. It is the first definite demonstration that a steroid can go through the "placental barrier."

This process, however, does not appear to be a simple diffusion. A rather constant relationship was found to exist between the levels of plasma 17-OH-CS in the mother and fetus at birth. In most instances, irrespective of the method of delivery and whether ACTH or compound F was administered to the mother before delivery, the values in cord plasma were 20 to 50 per cent of those in the mother. It seems highly improbable that the lower levels in the infant may have been due to a slow equilibration between maternal and fetal circulations, or to a rapid destruction of 17-OH-CS by the fetus. Where the maternal values were the lowest, the ratio had a tendency to be smaller, and thus, it would seem that a certain threshold level is necessary in the maternal circulation before this transmission can occur to any significant extent.

The intravenous infusion of ACTH into patients selected for cesarean section raised their 17-OH-CS levels in a usual manner, but it did not alter the maternal: fetal ratio. This observation can be explained by the fact that either ACTH does not cross the "placental barrier," the fetal levels being merely the reflection of maternal 17-OH-CS concentrations, or that passage across the placenta occurs, but the fetal adrenals do not respond as well as those of the mother. The first hypothesis seems to be more likely in view of the results obtained following compound F infusion.

It would appear from this study that little or no 17-OH-CS are produced by the fetal adrenal cortex, at least at the end of the third trimester of pregnancy. The levels in cord plasma found in cases of vaginal delivery seem to be conditioned by the high values in the mother; in contrast, following cesarean section, an apparently less stressful situation, the cord values are significantly lower. These observations are consistent with the finding that little or no plasma 17-OH-CS are detectable in the first few days of life and that normal adult levels are not achieved until after the first week of life (6).

If the fetal adrenal cortex does not produce any corticosteroids, the placenta and/or the maternal adrenals appear to be the most likely sources of 17-OH-CS in the fetal blood, although there is no evidence against the ovary as a source of the increased steroids. If the placenta synthesizes such hormones, one would have to assume from the data presented that the maternal side of the placenta functions differently from the fetal side. There are no known facts at present to suggest such a differentiation of the placenta but this possibility cannot be disregarded.

The increase in plasma and urinary corticosteroids reported in pregnant Addisonian patients would suggest that the maternal adrenals are not the only site of production of 17-OH-CS during pregnancy. Progesterone has been isolated from human placenta (20, 21) and one might wonder whether this steroid cannot be a precursor of the corticosteroids found in the pregnant Addisonian patients.

In contrast to the 17-OH-CS, the 17-ketosteroid values in cord plasma are higher than the maternal levels (8, 22). Both plasma and urinary 17-ketosteroids of the newborn tend to fall gradually during the first few weeks after birth, and do not rise again until the onset of puberty (22). Accordingly, the fetus would appear to be producing primarily 17-ketosteroids and little, if any, 17-OH-CS at term.

SUMMARY

In cases of vaginal delivery, labor increased the maternal level of plasma 17-OH-CS over the already elevated value found in the pregnant patient at term. In addition, delivery itself had a tendency to further raise the level.

The surgical trauma associated with cesarean section was probably responsible for the increase observed during that operation; the levels, however, were not as elevated as those noted in the vaginal series.

The concentrations of 17-OH-CS in cord plasma were approximately one-half to one-fifth of those of the corresponding mother in cases of vaginal delivery, and one-fifth, or a lesser fraction, of

those of mothers undergoing "elective, repeat cesarean section."

The infusion of 25 mg. of ACTH over a period of six hours produced a normal increase of the maternal levels.

The intravenous administration of compound F to mothers just prior to an elective cesarean section demonstrated that, near the end of gestation, this steroid can go through the "placental barrier" but in a definite proportion, the maternal concentration being the determining factor.

From the data presented, it is suggested that, at term, little or no 17-OH-CS are produced by either the fetal adrenal or the fetal side of the placenta. The most probable sites of biosynthesis of such hormones are the maternal adrenals and/or the maternal side of the placenta.

ACKNOWLEDGMENTS

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THE EFFECT OF GLUCAGON ON CARBOHYDRATE METABOLISM IN NORMAL HUMAN BEINGS [1]

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Since the isolation of glucagon (hyperglycemic-glycogenolytic factor, HGF) from the pancreas (1, 2), and the demonstration that it is released in appreciable amounts into the blood stream (3–5), there has been much speculation about the possibility that diabetes mellitus may result from an imbalance between secretion of insulin and glucagon (6–8). This speculation has been given additional weight by the demonstration that anterior pituitary growth hormone, a well established diabetogenic agent, causes release of a glucagon-like substance from the pancreas of experimental animals (5). The present investigation was undertaken to discover whether glucagon could produce an elevated blood glucose concentration with inhibition of glucose utilization, since this combination is usually observed in diabetic patients (9–11). These studies confirm and extend previous observations (12) that glucagon does not interfere with the uptake of glucose by the peripheral tissues.

METHODS

Arterial and venous samples were obtained from Cournand needles inserted into the antecubital vein and brachial artery after local infiltration with Procaine, without epinephrine. After allowing several minutes to elapse, samples were removed from artery and vein simultaneously. The interval between arterial and venous samples was never greater than thirty seconds.

Blood samples for glucose analysis were collected in fluoride-oxalate and deproteinated within a few minutes. Glucose was determined in quadruplicate by the Nelson modification of the Somogyi technique (13), using the specific precautions previously described to insure maximum reproducibility (14).

Serum inorganic phosphorus determinations were made on venous samples according to the method of Fiske and Subbarow (15).

Alpha amino nitrogen content of plasma and whole blood was determined on venous samples according to the method of Frame, Russell, and Wilhelmi (16, 17).

The concentration of blood ketone bodies was determined on arterial samples by a modification (18) of the method of Greenberg and Lester (19).

In acute experiments, three control pairs of arteriovenous samples were drawn and then 1.0 ml. (0.95 mg.) of glucagon [3] was immediately injected through the venous Cournand needle. Samples were taken 1, 2, 3, 5, 7, 10, and 15 minutes after the injection. A control experiment was run in two cases, by repeating the entire procedure, substituting a slow intravenous drip of 5 per cent glucose in water for glucagon. The rate of administration of glucose was adjusted to produce an arterial glucose curve approximating that produced by glucagon. The control experiments followed the glucagon experiments by at least one week.

The effect of HGF on the peripheral circulation was studied by following skin temperatures and finger plethysmographs in two volunteers for fifteen minutes after the injection of glucagon. The subjects rested in a constant temperature room at 82 degrees F. for twenty-four minutes before the injections. Skin temperatures were measured in seven locations by thermocouples; relative blood flow was estimated by finger plethysmography, using the technique of Burch (20). No blood chemical determinations were made in these subjects.

In a second group of subjects, 0.04 mg. per Kg. of glucagon was administered in 240 to 300 ml. of 0.9 per cent saline intravenously at a constant rate over the course of two hours. Control arterial and venous samples were drawn five minutes before and at the start of the glucagon infusion, and 15, 30, 60, and 120 minutes thereafter, while the glucagon was being administered. In

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[2] This work represents a portion of the material presented in satisfaction of the thesis requirement for the M.D. degree at the Yale University School of Medicine.

[3] The glucagon preparation employed in these experiments, kindly supplied by Dr. W. R. Kirtley, was Lilly Lot number 208-158 B-214, containing 0.95 mg. per ml. of protein in insulin diluting fluid. The glucagon was approximately 50 per cent pure, based on the potency of crystalline glucagon (0.2 microgram per kilogram of body weight, injected intravenously into anesthetized cats, causes a maximum blood sugar rise of 30 to 40 milligrams per cent, ten to fifteen minutes after injection). The insulin content was between 0.005 and 0.05 units per ml. of solution.

some instances another venous sample was drawn after the completion of the infusion.

A third group of subjects received 200 to 300 ml. of 5 per cent glucose by slow intravenous injection instead of glucagon. Conditions of this experiment were identical to those of the second group, except that only venous samples were drawn.

In most experiments of the latter two groups, hematocrit determinations were made before and after the infusions.

All subjects were adult males who had been eating a diet containing at least 250 gm. of carbohydrate daily. They were either entirely well or in late convalescence and in none was there evidence or history of any disorder of carbohydrate metabolism. Experiments were run in the early morning, with all patients in the post-absorptive state.

RESULTS

In the acute group (Table IA), the arterial glucose concentration began to rise within one minute after the injection of glucagon in four of the six subjects, and in a fifth the rise became apparent within four minutes. In one subject (W. P.) glucagon produced no change.

The concentration of glucose in the vein began to rise at almost the same time as in the artery, but the rise was somewhat less steep, so that in most instances there was a pronounced increase of arterio-venous difference within two to five minutes. By ten minutes there was a highly significant ($p < .01$) rise of the mean A–V glucose difference, and this was sustained throughout the experiment. There was no increase of A–V glucose difference in the single subject whose arterial glucose concentration failed to rise.

When glucose was given intravenously (Table IB), the arterial concentration rose gradually, and the arterio-venous glucose also increased, in a manner indistinguishable from that seen after glucagon.

TABLE I

A. Acute effect of glucagon on the arterial and venous glucose concentration of human beings

Name / Age / Clinical status	R. F. 27 Mentally defective			F. M. 65 Old cerebral thrombosis			W. P. 63 Old cerebral thrombosis			F. K. 25 Normal			E. K. 74 Old cerebral thrombosis			J. M. 56 Old cerebral thrombosis		
Minutes after glucagon	Glucose concentration			Glucose concentration			Glucose concentration			Glucose concentration			Glucose concentration			Glucose concentration		
	Art.	Ven.	A-V	Art.	Ven.	A-V	Art.	Ven.	A-V	Art.	Ven.	A-V	Art.	Ven.	A-V	Art.	Ven.	A-V
	mg./100 ml.			*mg./100 ml.*			*mg./100 ml.*			*mg./100 ml.*			*mg./100 ml.*			*mg./100 ml.*		
−4	81	79	2	66	57	9	69	64	5	69	63	6	74	66	8	81	87	−6
−2	84	79	5	68	66	2	69	62	7	78	74	4	77	72	5	87	86	1
0	84	79	5	70	69	1	72	69	3	74	70	4	76	68	8	87	85	2
1	92	81	11	74	70	4	65	68	−3	79	73	6	74	71	3	89	83	6
2	88	84	4	90	76	14	74	69	5	89	82	7	75	72	3	99	87	12
3	88	89	−1	91	80	11	71	69	2	88	85	3	77	71	6	102	96	6
5	106	94	12	101	94	7	74	71	3	101	90	11	87	77	10	107	100	7
7	120	101	19	111	98	13	77	76	1	111	97	14	92	81	11	117	116	1
10	137	119	18	117	103	14	77	74	3	116	103	13	96	83	13	120	115	5
15	154	131	23	115	110	5	72	71	1	118	105	13	Not taken			146	134	12

B. Effect of glucose on the arterial and venous glucose concentration

Name / Age / Clinical status	F. M. 65 Old cerebral thrombosis			F. K. 25 Normal		
Minutes after glucose	Glucose concentration			Glucose concentration		
	Art.	Ven.	A-V	Art.	Ven.	A-V
	mg./100 ml.			*mg./100 ml.*		
−4	96	91	5	96	88	8
−2	85	82	3	98	87	11
0	104	83	21	96	91	5
1	111	102	9	96	88	8
2	114	103	11	103	93	10
3	120	104	16	106	96	10
5	123	110	13	107	101	6
7	130	113	17	112	107	5
10	139	114	25	115	107	8
15	141	119	22	125	113	12

TABLE II

Effect of two-hour intravenous infusions of HGF on the arterial and venous glucose concentration (mg. per 100 ml.)

Name / Age / Condition	D. C. 16 Peptic ulcer			J. O. 45 Cerebrovascular accident			W. O. 23 Normal			F. B. 45 Arteriosclerotic heart disease			J. K. 28 Convalescence from poliomyelitis		
Minutes after start of HGF	Art.	Ven.	A-V	Art.	Ven.	A-V	Art.	Ven.	A-V	Art.	Ven.	A-V	Art.	Ven.	A-V
−5	91	88	3	84	82	2	83	79	4	95	88	7	89	85	4
0	88	85	3	87	82	5	92	88	4	92	86	6	87	84	3
15	149	128	21	135	111	24	170	144	26	141	116	25	154	142	12
30	168	158	10	159	136	23	188	154	34	166	142	24	182	161	21
60	162	151	11	151	144	7	130	121	9	180	159	21	174	167	7
120	118	115	3	107	94	13	79	69	10	146	138	8	139	135	4
P.I.*	Not taken				90			48			130			123	

* Post infusion venous glucose determination, drawn within 10 min. after HGF. "Hypoglycemic" reactions were noted in subjects D. C., W. O., F. B., and J. K.

There were no significant changes in peripheral blood flow following acute glucagon injection.

A rapid rise of arterial and venous blood glucose levels with an accompanying increased arteriovenous difference occurred during the two-hour glucagon infusions (Table II). In all instances a later decrease in glucose levels occurred while HGF was still being administered. Indeed in four of the five subjects clinical hypoglycemic reactions were noticed 110 to 120 minutes after the beginning of the infusions. Post-infusion venous glucose levels, in these four cases, demonstrated a continuing fall in all instances and definite hypoglycemia in one instance. Intravenous glucose eliminated or ameliorated the reaction.

Venous blood sugar curves varied considerably in the subjects to whom glucose was administered over two hours. In three, a peak was reached at one-half or one hour, and there was a decline of 10 to 208 mg. per cent during the second hour, but in two others, levels continued to rise throughout the infusion.

The arterial glucose concentration was compared with the A–V difference for the acute HGF groups

TABLE III

Serum inorganic phosphorus levels (mg. per cent) during HGF and glucose infusions

	HGF					Glucose			
Name / Age	D. C. 16	J. O. 45	W. O. 23	F. B. 45	J. K. 28	W. O. 23	J. H. 66	J. M. 75	W. G. 21
Minutes after start of HGF									
−5	4.30	3.69	3.47	2.96	4.47	4.45	3.15	3.67	4.44
0	4.40	3.61	3.44	2.84	4.55	4.34	3.11	3.57	—
15	3.60	2.82	3.28	2.64	4.27	4.30	3.03	3.48	4.12
30	3.10	2.81	3.23	2.59	4.14	4.22	2.94	3.40	3.83
60	2.77	2.67	3.13	2.53	3.97	4.15	2.86	3.27	3.70
120	2.96	2.56	3.05	2.36	3.86	4.07	2.78	3.16	3.54
Max. decrease	1.63	1.05	.39	.48	.69	.27	.33	.41	.90
Max. decrease %	37.0	29.1	11.4	16.9	15.2	6.2	10.6	11.5	20.3

Effect of two-hour HGF intravenous infusions on blood ketones (mg. per cent acetone)

	HGF		
−5	1.32	0.23	0.56
0	0.84	0.47	
15	1.46	0.29	
30	1.04	0.82	
60	0.44	0.00	
120	2.02	0.41	0.77

TABLE IV

A. Plasma and whole blood alpha amino nitrogen levels during two-hour HGF infusions (mg. per cent)

	P	WB	P	WB	P	WB	P	WB	P	WB	P	WB	P	WB
Name	D. C.		J. O.		W. O.		F. B.		J. K.		J. H.		J. M.		W. G.	
Age	16		45		23		45		28		66		75		21	
Minutes after start of HGF																
−5	4.8	4.6	5.2	5.83	4.60	7.48	5.05		3.42	7.17						
0	4.9	4.4	5.1	6.14	4.50	7.30	5.05	10.55	3.31	6.94						
15	4.7	4.0	4.9	6.38	4.22	6.75	4.77	10.1	3.10	6.70						
30	4.4	3.9	4.7	5.85	4.10	6.45	4.67	9.6	2.98	6.27						
60	4.0	3.5	4.5	6.14	3.82	6.02	4.50	9.2	2.63	5.24						
120	3.6	3.2	4.4	5 95	3.68	5.62	4.35	9.05	2.32	5.16						
Max. decrease	1.3	1.2	.7	.29	.82	1.68	.70	1.05	.99	1.78						
Max. decrease %	26.6	27.3	13.8	4.7	18.2	23.0	13 9	10.0	30.0	25.6						

B. Plasma and whole blood alpha amino nitrogen levels during two-hour glucose infusions (mg. per cent)

−5					2.73	7.40					3.54	6.50	3.73	8.33	4.44	8.77
0					2.65	7.55					3.38	6.54	3.60	7.84	4.54	8.62
15					2.60	7.00					3.30	6.22	3.35	7.46		
30					2.55	6.55					3.13	5.68	3.25	7.24		
60					2.48	6.50					2.73	5.78	3.06	6.76		8.16
120					2.38	6.44					2.70	5.47	2.87	6.22	3.92	7.74
Max. decrease					.27	1.11					.68	1.07	.73	1.62	.62	.88
Max. decrease %					10.2	14.7					20.1	16.4	20.3	20.7	13.7	10.2

and for the two patients in whom A–V differences were determined after acute glucose administration. The slopes of the lines of the HGF ($b = .25$) and glucose ($b = .31$) groups were very similar ($p = .40$).

Serum phosphate levels declined in every case during HGF or glucose infusions (Table III). In both groups there was a similar gradual decrease with the lowest value at the end of the infusion (D.C. of the HGF group was an exception). The average percentage of maximum decrease during the infusions was 21.9 ± 9.7 per cent [4] in the HGF group and 12.4 ± 5.3 per cent in the glucose group.

Whole blood and plasma levels of alpha amino nitrogen showed a similar progressive decrease during HGF and glucose infusions (Table IV). The average percentages of maximum decrease noted were: 18.1 ± 10.0 per cent and 20.5 ± 7.5 per cent in the HGF group, and 15.5 ± 4.5 per cent and 16.1 ± 4.9 per cent in the glucose group, for whole blood and plasma levels, respectively.

Ketone body levels were determined in three of the subjects infused with HGF (Table III). The levels were not elevated initially and no significant changes were noted during the course of the infusions.

Hematocrit levels before and after infusions were determined in all subjects of the HGF group and in two of the glucose group. In no instance was there a change exceeding 1 per cent.

DISCUSSION

The fact that glucagon raises the blood glucose concentration has led a number of authors to suggest that it may be diabetogenic (6–8). Transient hyperglycemia, however, is not diabetes. In order to prove that a substance is truly diabetogenic it is necessary to show that, in addition to raising the blood glucose concentration, it also inhibits glucose utilization, either absolutely or relative to the arterial glucose concentration, or that it causes major dislocations of the metabolic pattern associated with hyperglycemia, such as ketonemia or acidosis. The present experiments were designed to determine whether the action of glucagon meets any of these criteria of a true diabetogenic agent.

[4] Throughout this communication, statistical variability is expressed as the standard deviation.

The simplest and most convincing evidence of altered glucose utilization is an actual change of peripheral glucose oxidation. We were unable to investigate this question directly. Good indirect evidence can be obtained from the arterio-venous glucose concentrations provided that no changes of peripheral blood flow occur simultaneously. We established that glucagon (unlike epinephrine) has no effect on peripheral blood flow. The arterio-venous glucose differences measured in these experiments are, therefore, direct reflections of alterations of peripheral glucose utilization (including entry into cells, phosphorylation, glycogen formation, and oxidation to pyruvate or lactate or to carbon dioxide). The validity of the arterio-venous glucose difference as an index of carbohydrate utilization is well established (9, 21, 22). In our studies, both intravenous glucose and glucagon raised the arterial blood glucose level and increased the arterio-venous difference in a parallel fashion. The relationship between arterial blood glucose concentration and arterio-venous difference after glucagon was the same as after glucose and closely resembles that found by Somogyi after oral glucose (21). No evidence whatever could be found to indicate that glucagon reduced peripheral glucose utilization in these experiments. These findings are like those of Van Itallie, Morgan, and Dotti (12), who gave similar doses of HGF in saline infusions during one-half hour periods to normal subjects in the post-absorptive state. They also noted a hyperglycemic effect with increased arterio (capillary)-venous glucose differences. When A–V differences after glucose administration were compared to those after HGF, their work suggested that HGF may have enhanced glucose utilization slightly.

A somewhat less specific index of glucose utilization is the change in serum inorganic phosphate after the administration of a test substance. There is abundant evidence that the administration of glucose or insulin to normal animals and human beings causes a decreased level of blood and serum inorganic phosphorus (23–26) and that this response is usually diminished or absent in diabetics (23, 24, 26). This response seems to be a function of the disappearance of hexose from the blood (23, 24, 27, 28). In our experiments, the serum inorganic phosphate fell at least as rapidly and as

far after glucagon as it did after glucose. Kirtley, Waife, Helmer, and Peck (29) also produced a fall of serum phosphate in normal subjects given glucagon. These observations do not support the concept that glucagon interferes with glucose utilization.

In our experiments, the plasma and whole-blood alpha-amino nitrogen fell during glucagon administration. This effect has little specific bearing on the rate of glucose utilization, but it is similar to the changes seen when glucose utilization is increased after the administration of glucose or insulin. Although glucagon reduced plasma amino nitrogen in normal human beings, growth hormone (claimed by some to act by causing a release of glucagon) had no such effect (30).

The administration of glucagon failed to alter the blood ketone body level, although the liver glycogen was probably greatly depleted toward the end of the two-hour infusion of HGF. In this respect also, glucagon differs from the growth hormone preparations (30, 31).

During the prolonged HGF infusions, the arterial glucose concentration uniformly reached a peak at 1 hour and then declined, often to such low levels or at so great a speed as to produce hypoglycemic symptoms. The falling phase probably represents a combination of two factors. The hyperglycemia provokes an increased secretion of insulin (4, 32, 33) so that disposal of the glucose into the peripheral tissues is accelerated (34, 35). This effect is illustrated in our patients (J. H., W. G.) who received glucose alone and whose glucose concentration began to drop while the glucose injection was continuing. Since little of the glucose entering the tissues is later released as glycogen precursors (29) the liver glycogen falls steadily. In this respect, glucagon is quite different from epinephrine which promotes the release of lactate from the muscles, and inhibits the peripheral utilization of glucose, thus permitting the maintenance of a normal liver glycogen level (12, 36). As a result of these two factors, liver glycogen becomes inadequate and the release of glucose from the liver falls behind the rate of peripheral disposal. To test this hypothesis, a second large dose of glucagon (.95 mg. I.V.) was given suddenly to a subject whose arterial glucose level was falling during HGF infusion. The second dose of glucagon did not halt or reverse the falling glucose level.

The late hypoglycemic effect of glucagon is not caused by the small amount of insulin it contains, since the total amount of insulin received by our subjects could not have exceeded 1 unit in 2 hours, or .05 units in a single injection. Moreover, the late hypoglycemia can be produced simply by injecting glucose alone, without exogenous insulin.

Our finding that glucagon does not interfere with the peripheral utilization of glucose in human beings is in agreement with most of the studies of other investigators using animals as subjects (37–39).

Although there is no evidence that glucagon can reduce glucose utilization *in vivo*, it apparently can reduce the glycogenic effect of insulin on diaphragm muscle *in vitro* (40, 41). The glycogen metabolism of the diaphragm, however, is not characteristic of other voluntary muscle (42), and the pertinence of studies of such special tissues as diaphragm to the over-all body metabolism is not clear.

SUMMARY AND CONCLUSIONS

Glucagon (pancreatic hyperglycemic-glycogenolytic factor) was given intravenously to normal human subjects. Acutely, it produced a rapid rise of arterial and venous blood glucose concentrations, with increased arterio-venous glucose differences. No change in peripheral blood flow occurred.

When given by constant intravenous infusion for two hours, glucagon caused a rise of both arterial and venous glucose concentrations with increasing arterio-venous glucose differences, until about 1 hour, after which the arterial glucose level fell. Toward the end of the test period hypoglycemic symptoms often occurred. The falling glucose concentration could not be prevented by larger doses of glucagon.

Over the two-hour period serum inorganic phosphate and plasma and whole blood alpha amino nitrogen concentrations fell steadily. There was no change in blood ketone body concentration. In all respects, the effects of HGF were similar to those of doses of glucose which produced comparable arterial glucose concentration curves, and different from those of growth hormone.

These observations give no support to the con-

cept that glucagon is diabetogenic, since no evidence was obtained of reduced glucose utilization. It is more likely that, in the intact organism, glucagon works synergistically with insulin to release glucose from the liver, thus making it available for the peripheral utilization which is promoted by insulin.

Addendum

Elrick, Hlad, and Witten (43) have recently found that the A–V glucose difference after glucagon is greater than during comparable hyperglycemia produced by glucose alone. These findings are somewhat at variance with ours, but the difference may reflect the fact that in Elrick, Hlad, and Witten's studies the patients were heavily loaded with glucose before the administration of glucagon. In any case, no eivdence of inhibition of glucose utilization was found.

ACKNOWLEDGMENTS

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GASTROINTESTINAL WATER AND ELECTROLYTES. I. THE EQUILIBRATION OF RADIOSODIUM IN GASTROINTESTINAL CONTENTS AND THE PROPORTION OF EXCHANGEABLE SODIUM (Na_e) IN THE GASTROINTESTINAL TRACT [1]

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The concept of the partition of body water and electrolytes into two anatomical entities, extracellular and intracellular fluid, is well established. This is based on their fundamental differences in electrolyte structure (1). More refined definitions of water and electrolyte anatomy have become possible by the application of tracer dilution methods to *in vivo* estimation of body water and electrolyte content (2, 3). Thus, ideas on the organization of extracellular fluid have undergone distinct evolution in recent years. It has been pointed out that the equilibration properties of sodium in bone and of inulin and thiosulfate in dense connective tissue distinguishes the extracellular phase of these tissues from free interstitial fluid (4, 5). The proposal has also been made that the term transcellular fluid be applied to those extracellular fluids which are not in simple diffusion equilibrium with plasma and are probably formed as a result of active cellular transport mechanisms (3). This would include fluid in the gastrointestinal tract, joint spaces, synovial sacs, cerebrospinal canal, anterior chamber of the eye, glandular ducts and acini, and in the renal tubules. The physiological significance of transcellular fluids will depend, in part, on the volume of these fluids and the extent of exchangeability of their individual components.

This and the three articles to follow (6–8) will report the results of studies on the magnitude of intraluminal gastrointestinal water and electrolytes in rabbits and humans and on the equilibration of gut water and electrolytes with body water and electrolytes, using tracer indicators, in rabbits. These studies were undertaken because a) the gastrointestinal contents probably contribute considerably to the volume of transcellular fluid, and b) it has been shown that there is rapid bi-directional transport of water and electrolytes between blood and the intestinal lumen (9). In some commonly used laboratory animals, such as the rabbit and guinea pig, intraluminal gastrointestinal water content estimated by desiccation methods constituted a major subdivision of body water (10).

This communication presents observations on a) the amount of intraluminal gastrointestinal sodium relative to total exchangeable sodium (Na_e) and the extent of radiosodium exchange equilibrium in gastrointestinal contents in rabbits and b) the amount of intraluminal gastrointestinal sodium in human cadavers.

METHODS

A. Rabbits

Forty adult albino rabbits were studied in pairs consisting of a male and a non-gravid female. The animals were allowed to consume pellets and water *ad libitum* until the time of injection, or else water was allowed *ad libitum* but food was withheld for a variable period of time before sacrifice. The average sodium intake prior to fasting was 15 mEq. per day. Figures 1 and 2 depict the periods of fasting up to the time of sacrifice.

All animals were injected intraperitoneally with 100 to 150 microcuries of $Na^{22}Cl$ [3] from calibrated syringes. The injected solution was made isotonic with NaCl, sterilized and neutralized to pH 7.0. Isotopic purity was evaluated by prolonged observations of the rates of decay on aliquots taken from each shipment. At the time of radioassay there was less than 8 per cent contamination

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[2] Established Investigator of the American Heart Association.

[3] Prepared by deuteron bombardment of $Na^{22}Cl$ in the 60 inch cyclotron in the Crocker Radiation Laboratory, University of California, Berkeley.

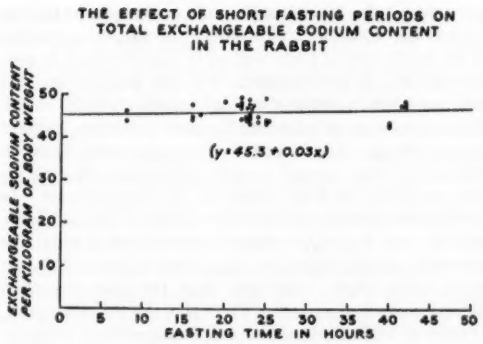
THE EFFECT OF SHORT FASTING PERIODS ON TOTAL EXCHANGEABLE SODIUM CONTENT IN THE RABBIT
EXCHANGEABLE SODIUM CONTENT PER KILOGRAM OF BODY WEIGHT
(y=45.3+0.03x)
FASTING TIME IN HOURS

FIG. 1. THE EFFECT OF FASTING PERIODS OF 8 TO 44 HOURS ON THE TOTAL EXCHANGEABLE SODIUM CONTENT IN RABBITS

Water was allowed *ad libitum*. The line drawn through the experimental values was determined by the method of least squares.

with P^{32}. The scintillation counting technique employed, however, detects virtually none of this contaminant since P^{32} electrons are absorbed by the shield in this system. Urine and stool passed during the period of isotope equilibration were collected in metabolism cages. The rabbits were anesthetized with 2 ml. of 2 per cent Nembutal® administered into a dorsal ear vein 20 to 26 hours after injection of radiosodium and then were weighed to the nearest gram. Cardiac blood was drawn by puncture through the intact chest wall. The abdominal cavity was opened, and all bladder urine was aspirated and pooled with the cage urine. Double ligatures were placed at the cardia of the stomach, pylorus, ileocecal valve and the mid-transverse colon. The intact serosal surfaces were washed with distilled water and carefully dried to prevent contamination of the pans with blood. The rubber gloves worn by the experimenters were carefully washed off with distilled water. Each segment of the gastrointestinal tract was removed separately, opened along the long axis, and the contents were carefully emptied into distilled water-rinsed containers. The mucosal surfaces were stripped as gently as possible, but a thin layer of mucus invariably clung to the mucosal surface. Absolute recovery of gastrointestinal contents was not attempted in order to avoid bleeding or epithelial damage. The evacuated contents were transferred quantitatively to graduated cylinders with multiple rinses using more than 100 ml. of distilled water in every instance. The volumes were recorded, and aliquots were filtered through glass wool. Aliquots of serum, gastrointestinal contents, urine and stool were taken for radioactive assay and chemical analysis.

One-ml. aliquots of serum, gastrointestinal contents and urine were plated in triplicate on filter paper, dried under an infra-red lamp and covered with parafilm. Three separate dilutions of the injected solution were each plated in triplicate and processed as above. Radioactivity assays were carried out with an aluminum-covered end-window, thallium-activated sodium iodide scintillation counter and autoscaler. Preliminary observations revealed that self-absorption loss was not encountered up to twice the quantity of solids present in the experimental samples and that coincidence loss was not detected up to five times the counting rates of the experimental samples. All samples, however, were corrected for background and radioactive decay ($T_{1/2} = 15.04$ hours [11]). A minimum of 5,000 counts were collected on each planchet.

Triplicate sodium analyses were made from duplicate dilutions on aliquots of serum, gastrointestinal contents, urine and stool with a lithium internal standard flame photometer (Baird Associates—Na II filter). Since random samples of gastrointestinal contents contained less than 12 mEq. per liter of calcium, a correction for the contribution of calcium emission to sodium assays was unnecessary.

B. Human subjects

Thirteen human subjects were studied at the time of postmortem examination. The gross and microscopic postmortem findings, as well as the age, sex, body weight and height of each subject, were recorded. Patients with demonstrable edema or organic gastrointestinal disease were eliminated from the study.

Double ties were placed at the cardio-esophageal junction, the pylorus of the stomach, the ileocecal valve, and just distal to the hepatic flexure of the transverse colon. The tract was separated into segments by cutting between ties and removed from the peritoneal cavity. The serosal surfaces were trimmed, washed with distilled water, carefully dried, and each segment of the tract weighed on a torsion balance to within 1.0 gram. Each segment of the tract was opened along its long axis. The mucosal surfaces were stripped with a gloved finger, and the contents transferred to distilled water-cleaned

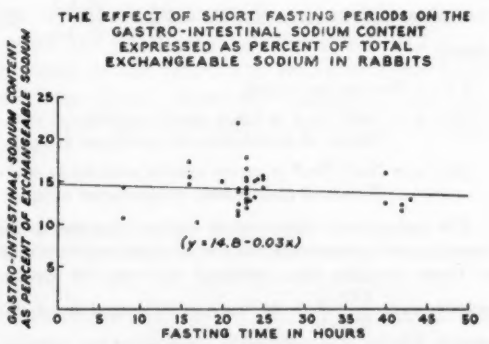
THE EFFECT OF SHORT FASTING PERIODS ON THE GASTRO-INTESTINAL SODIUM CONTENT EXPRESSED AS PERCENT OF TOTAL EXCHANGEABLE SODIUM IN RABBITS
GASTRO-INTESTINAL SODIUM CONTENT AS PERCENT OF EXCHANGEABLE SODIUM
(y=14.8-0.03x)
FASTING TIME IN HOURS

FIG. 2. THE EFFECT OF FASTING INTERVALS ON THE GASTROINTESTINAL SODIUM CONTENT OF RABBITS RELATIVE TO THE TOTAL EXCHANGEABLE SODIUM CONTENT

Water was allowed *ad libitum*. The line drawn through the experimental values was determined by the method of least squares.

pans, using multiple distilled water rinses. The wall of each portion of the tract was carefully dried and reweighed. A thin layer of mucus invariably clung to the mucosal surfaces at the end of these manipulations. The gastrointestinal contents were homogenized, the total volume recorded using graduated cylinders, and aliquots were taken for estimation of total solids, chloride, sodium and potassium.

Triplicate sodium analyses were made from duplicate dilutions of the separate gastrointestinal contents with a lithium internal standard flame photometer (Baird Associates—Na II filter).

CALCULATIONS

A. Rabbits

The total exchangeable sodium content (Na_e) was calculated from the dilution formula (2):

$$Na_e = \frac{Na_i^{24} - Na_o^{24}}{Na_s^{24}/Na_s^{22}} \qquad (1)$$

where:

Na_i^{24} = injected radiosodium in counts per minute.

Na_o^{24} = radiosodium excreted in the urine (bladder plus cage urine) to time of equilibrium blood sample.

Na_s^{24}/Na_s^{22} = serum specific activity at equilibrium of distribution in counts per mEq.

In these studies the equilibrium samples were obtained 20 to 26 hours after administration of the radiosodium solution. Urinary loss of radiosodium during this period averaged 6.6 per cent of the injected amount, with a range of 2.2 to 10.7 per cent.

The degree of ionic exchange of intraluminal sodium with serum sodium was evaluated by relating gastrointestinal specific activities to the serum specific activities. The fraction of the tissue element, in this case intraluminal sodium, exchanged with serum element is given by the formula:

$$\text{Specific activity ratio (S.A.R.)} = \text{F.E.} = \frac{(S.A.)_t}{(S.A.)_s} \qquad (2)$$

where:

F.E. = fraction exchanged.

$(S.A.)_t = Na_t^{24}/Na_t^{22}$ = tissue specific activity at equilibrium of distribution in counts per mEq.

$(S.A.)_s = Na_s^{24}/Na_s^{22}$ = serum specific activity at equilibrium of distribution in counts per mEq.

The derivation of this formula requires that the serum specific activity be constant or almost constant at the time of tissue sampling (4). Although equating the specific activity ratio $\frac{(S.A.)_t}{(S.A.)_s}$ to the fraction exchanged is not rigorously precise, it is sufficiently accurate for the purposes of this study.

B. Human subjects

The intraluminal gastrointestinal sodium content has been expressed as an absolute quantity (mEq.), as the amount per unit of body weight (mEq. per kgm.) and as a per cent of the "predicted" total exchangeable sodium content (*cf.* Table VI). To reduce the data to a fraction of the labile pool of body sodium it was necessary to predict the Na_e in each subject. For this purpose we used the previously published normal standards (12), rather than attempting to take into account deviations induced by a particular disease state. The mean value listed in these tables was applied to each patient according to sex and age, using the body weight as the reference unit. In studies on patients with a wide variety of disease states, the Na_e was invariably found to be less than twice the expected normal values in edema-free subjects (13). It seems most likely, therefore, that the gastrointestinal sodium content expressed as per cent of the Na_e is accurate, at least to within a factor of 2 as a postmortem estimate. The absolute error resulting from agonal or postmortem migration of sodium across the gastrointestinal mucosa is unknown. The method employed has the advantage of eliminating the possibility of weighting the data by arbitrary selection of a predicted Na_e value.

RESULTS

A. Rabbits

To evaluate the possibility of sex-linked differences in either total exchangeable sodium content or the relative amount of intraluminal gastrointestinal sodium content, the rabbits were studied in pairs consisting of a male and a non-gravid female. The pairs of animals were subjected to identical fasting periods. Table I details comparisons of the Na_e, Na_e per kgm. of body weight and the "total" gastrointestinal sodium between male and female rabbits matched for age and body weight. "Total" gastrointestinal sodium refers to the intraluminal sodium content of the gastroin-

TABLE I

The exchangeable sodium (Na_e) and gastrointestinal sodium content of male versus female rabbits

	Male	Female	t	p
Number	16	16		
Body weight in kgm. ±s.d.*	2.14±0.20	2.09±0.12	0.83	>0.4
Na_e in mEq. ±s.d.	96.5 ±6.5	96.5 ±7.3	0	1.0
Na_e/body weight in mEq./kgm. ±s.d.	45.3 ±2.3	46.2 ±2.3	1.07	>0.2
"Total" G-I sodium† as % of Na_e ±s.d.	14.0 ±2.1	14.3 ±2.4	0.37	>0.7

* s.d. $= \sqrt{\frac{\Sigma(x - \bar{x})^2}{n}}$.

† "Total" G-I sodium refers to the intraluminal sodium content of the gastrointestinal tract from the cardia of the stomach to the mid-transverse colon.

testinal tract from the cardia of the stomach to the mid-transverse colon. The p values for either body sodium content or the proportion of body sodium within the gastrointestinal tract do not achieve statistical significance. Because of these findings subsequent tabulations were made without regard to the sex of the individual animals.

Since the quantity of sodium in the gastrointestinal contents could be a function of food intake either by direct addition or by stimulation of gastrointestinal secretions, the effect of short fasting periods on the Na_e and "total" gastrointestinal sodium expressed as per cent of Na_e was investigated; the results are depicted in Figures 1 and 2. The periods of fasting varied from 8 to 43 hours. Within this interval as judged by the slope "λ," there is an insignificant effect on Na_e ($\lambda = +0.03$ per hour) and on the gastrointestinal fluid sodium content ($\lambda = -0.03$ per hour). These data indicate that short fasting periods insured basal gut fluid sodium contents without depletion of body sodium or shifts of sodium from the lumen of the gastrointestinal tract to the carcass.

Table II enumerates two fundamental parameters of body sodium anatomy, namely, the serum sodium concentration and the exchangeable sodium content. Serum sodium concentrations averaged 145 ± 4 mEq. per liter, essentially the same as in humans. Exchangeable sodium content averaged 46.0 ± 2.0 mEq. per kgm. of body weight, whereas in humans the Na_e averages 41.0 ± 5 mEq. per kgm. (12).

Exchange equilibrium between gastrointestinal sodium and body sodium was evaluated by expressing the specific activity of gut fluid as a fraction of the serum specific activity (*cf.* Table III). The values of 0.95 ± 0.128 for stomach, 0.97 ± 0.045 for small intestine, 0.99 ± 0.049 for cecum and transverse colon, and 0.99 ± 0.036 for the "total" gastrointestinal tract indicate that all of the gut sodium is freely exchanged with extracellular sodium. The small differences from unity were evaluated by the formula:

$$t = \frac{1.00 - \bar{x}}{\text{s.e.}} \qquad (3)$$

where:

$\bar{x}$ = mean value
s.e. = standard error

The p value for small intestine sodium exchange is < 0.02 and does achieve statistical significance. However, the quantity of sodium involved (3 per cent) is negligible.

The magnitude of intraluminal gastrointestinal sodium content is summarized in Table IV. "Total" gastrointestinal sodium is 14.2 ± 2.4 per cent of the Na_e; with the smallest fraction in the stomach, 0.9 ± 0.4 per cent of the Na_e, an intermediate amount in the small intestine, 3.2 ± 1.2 per cent of the Na_e, and the major portion in the cecum and proximal transverse colon, 10.2 ± 2.1 per cent of the Na_e. The proximal segment of the large bowel, therefore, contains 72 per cent of the total gastrointestinal sodium in the normal rabbit.

TABLE II

The exchangeable sodium (Na_e) content in the rabbit *

	Body weight *kgm.*	Serum Na *mEq./L.*	Exchangeable Na *mEq.*	Exchangeable Na *mEq./kgm.*
Number	35	35	35	
Mean	2.12	145	97.4	46.0
s.d.	±0.21	±4	±7.1	±2.0
Coefficient of variation	10.0%	2.8%	7.3%	4.3%

* Fasting period varied from 8 to 43 hours prior to sacrifice.

TABLE III

*The equilibration of Na^{24} with intraluminal gastrointestinal sodium in the rabbit**

	Number	Fasting period *hours*	Specific activity ratio† Mean	s.d.	t	p
Stomach	17	20–24	0.95	±0.128	1.61	>0.1
Small intestine	17	20–24	0.97	±0.045	2.74	>0.01 <0.02
Cecum and transverse colon	17	20–24	0.99	±0.049	0.84	>0.4
"Total" G-I	35	8–43	0.99	±0.036	1.15	>0.2

* $Na^{24}Cl$ was injected intraperitoneally 20 to 25 hours prior to sacrifice.
† This ratio was obtained by dividing the specific activity of the gastrointestinal contents by the specific activity of the serum.

TABLE IV

The intraluminal gastrointestinal sodium content in the rabbit

	Stomach		Small intestine		Cecum and transverse colon		"Total" G-I*	
	mEq.	*% of Na_e*	*mEq.*	*% of Na_e*	*mEq.*	*% of Na_e*	*mEq.*	*% of Na_e*
Number	17		17		17		35	
Fasting period in hours	20–24		20–24		20–24		8–43	
Mean	0.8	0.9	3.1	3.2	10.0	10.2	13.7	14.2
s.d.	±0.4	±0.4	±1.1	±1.2	±2.0	±2.1	±2.4	±2.4
Coefficient of variation	50%	44%	35%	37%	20%	20%	18%	17%

* "Total" G-I sodium refers to the intraluminal sodium content of the gastrointestinal tract from the cardia of the stomach to the mid-transverse colon.

The impressive amount of sodium in the cecum and proximal transverse colon raises the possibility of fairly large rates of sodium loss in the stool. This question was examined by measuring the intraluminal sodium content of the descending colon and rectum and by quantitative 24-hour stool collections during short fasting periods (16 to 41 hours). The results are listed in Table V. The distal large bowel had a mean sodium content of 1.9 per cent of the "total" gastrointestinal sodium content, and the rate of stool sodium excretion averaged 0.40 mEq. per day, which is equivalent to 2.9 per cent of the "total" gastrointestinal sodium or 0.41 per cent of the Na_e. The cecal mechanism for sodium conservation is apparently quite efficient.

B. Humans

The results obtained on postmortem examination are listed in Table VI. The interval from the time of death to the time of collection of gastrointestinal contents varied from 6 to 22 hours. Perusal of the data indicates that there is no gross relation between the quantity of gastrointestinal sodium and the interval between death and postmortem examination. The smallest quantity of gastrointestinal sodium (15.4 mEq.) was found in a patient with an 11-hour postmortem interval; the largest amount (70.5 mEq.) was found in a patient with a 12-hour postmortem interval. This series consisted of 13 patients, 8 males and 5 females, varying in age from 50 to 77 years. The clinical and pathological features of these cases are summarized in the appendix.

TABLE V

The sodium content of the distal colon and the rate of stool sodium excretion in the rabbit

	Descending colon and rectum		Stool sodium per 24 hours	
	Total mEq.	*% of "Total" G-I sodium*	*Total mEq.*	*% of "Total" G-I sodium**
Number	8		16	
Fasting period in hours	22–41		16–41	
Mean	0.28	1.9	0.40	2.6
Range	0.06–0.56	0.6–4.1	0–1.47	0–8.2

* "Total" G-I sodium refers to the intraluminal sodium content of the gastrointestinal tract from the cardia of the stomach to the mid-transverse colon.

To express the intraluminal gastrointestinal sodium content in terms of the total labile pool of body sodium, a predicted Na_e was calculated for each case from previously published normal values (12), taking into account the patient's age, sex and body weight. Mean "total" gastrointestinal sodium was 0.54 mEq. per kgm. of body weight, or 1.6 per cent of the predicted Na_e. Stomach and proximal large bowel sodium contents almost equaled each other, averaging 0.18 mEq. per kgm. of body weight, or 0.45 per cent of the predicted Na_e, and 0.14 mEq. per kgm. of body weight, or 0.36 per cent of the predicted Na_e, respectively. Small bowel contents contained the largest fraction of gut sodium with a mean value of 0.30 mEq. per kgm. of body weight or 0.74 per cent of the predicted Na_e.

DISCUSSION

Sodium is universally present, usually in high concentrations, in body secretions. In humans the lowest concentrations of sodium tend to occur in stomach and cecal aspirates, averaging 60.4 and 52.5 mEq. per liter, respectively, while the highest concentrations occur in biliary and pancreatic secretions, averaging 148.9 and 141.1 mEq. per liter, respectively (14). Nevertheless, sodium in the gastrointestinal tract has been ignored

TABLE VI

Intraluminal gastrointestinal sodium content in the human subject studied postmortem

Sex	Pathological diagnosis	Post-mortem interval *hours*	Age *yrs.*	Body weight *kgm.*	Predicted Na_e *mEq.*	Stomach			Small intestine			Cecum and proximal transverse colon			"Total" G-I		
						mEq.	*mEq./ kgm.*	*% of Na_e*	*mEq.*	*mEq./ kgm.*	*% of Na_e*	*mEq.*	*mEq./ kgm.*	*% of Na_e*	*mEq.*	*mEq./ kgm.*	*% of Na_e*
F-1	Recent myocardial infarction	6	77	35.0	1,380	4.7	0.14	0.34	9.9	0.28	0.72	4.6	0.13	0.33	19.2	0.55	1.4
M-2	Rheumatic heart disease with mitral stenosis	11	53	51.8	2,120	4.4	0.08	0.21	8.8	0.17	0.41	2.3	0.04	0.11	15.4	0.30	0.7
M-3	Hypertensive cardiovascular disease	22	51	43.6	1,890	12.0	0.28	0.63	11.4	0.26	0.60	2.7	0.06	0.14	26.1	0.60	1.4
F-4	Pituitary tumor	9	54	58.2	2,300	2.5	0.04	0.11	18.8	0.32	0.82	3.1	0.05	0.14	24.4	0.42	1.1
M-5	Squamous cell carcinoma of lung	7	64	43.6	1,780	22.2	0.51	1.25	14.4	0.33	0.81	4.9	0.11	0.28	41.5	0.95	2.3
F-6	Cerebral arteriosclerosis with encephalomalacia	6	56	58.2	2,300	17.6	0.30	0.77	8.4	0.14	0.37	18.7	0.32	0.81	44.7	0.77	1.9
F-7	Hypertensive and arterio-sclerotic heart disease	22	68	63.4	2,500	2.7	0.04	0.11	17.7	0.28	0.71	6.6	0.10	0.26	27.0	0.43	1.1
M-8	Paralysis agitans and bronchopneumonia	16	69	63.2	2,590	4.5	0.07	0.17	36.4	0.58	1.40	21.5	0.34	0.83	62.3	0.99	2.4
F-9	Cerebral thrombosis	20	67	65.0	2,570	25.6	0.39	1.00	9.6	0.15	0.37	0	0	0	35.2	0.54	1.4
M-10	Dissecting aneurysm of ascending aorta	19	59	68.0	2,780	1.7	0.02	0.06	18.2	0.27	0.66	1.6	0.02	0.06	21.4	0.32	0.8
M-11	Recurrent myocardial infarction	16	72	64.5	2,640	6.5	0.10	0.25	3.7	0.06	0.14	14.9	0.23	0.56	25.1	0.39	1.0
M-12	Chromophobe adenoma of pituitary	7	50	70.0	2,860	12.9	0.18	0.45	22.2	0.32	0.78	8.2	0.12	0.29	43.3	0.62	1.5
M-13	Traumatic demyelinization of cervical spinal cord	12	76	54.5	2,230	10.9	0.20	0.49	40.8	0.75	1.83	18.8	0.34	0.84	70.5	1.29	3.2
					Mean:	9.9	0.18	0.45	16.9	0.30	0.74	8.3	0.14	0.36	35.1	0.54	1.6
					Range:	1.7– 25.6	0.02– 0.39	0.06– 1.25	3.7– 40.8	0.06– 0.75	0.14– 1.83	0– 21.5	0– 0.34	0– 0.84	15.4– 70.5	0.30– 1.29	0.7– 3.2

in past considerations of body water anatomy. This is in spite of the fact that in many species the volume of intraluminal gastrointestinal fluid is considerable (10, 15).

The justification for separate identification of gut lumen contents rests firmly on both anatomical and physiological grounds. Anatomically, gastrointestinal sodium is clearly extracellular and falls in the same general category as plasma and interstitial fluid. However, the similarity ends there. Visscher and his co-workers have shown that the intraluminal gut-plasma concentration ratios for sodium, chloride and hydrogen ions may exceed 100; that water may enter the intestinal phase against a concentration gradient; that sodium may leave the intestinal phase against a concentration gradient; that the total flux of sodium ion across the intestinal mucosa is higher at the oral end; and that the flux of sodium out of the intestinal lumen relative to inward flux is greatest at the aboral end of bowel (9, 16–18). These findings indicate that exchange across the gastrointestinal mucosa probably involves active transport mechanisms and is not entirely a function of simple diffusion or pressure gradients. The decreased transport of sodium from ileal loop to blood during adrenal cortical insufficiency (19) and the decreased gastrointestinal sodium content in ACTH-treated, fasted, nephrectomized rats (20) support this conclusion, since hormonal influences on active transport mechanisms are well known.

The gastrointestinal specific activity ratios of 0.95, 0.97, 0.99 and 0.99 for stomach, small intestine, proximal large bowel and "total" gastrointestinal contents, respectively, confirms the complete exchangeability of gastrointestinal sodium. This phase of sodium is, therefore, an integral part of the body pool of labile sodium. The lability of gastrointestinal sodium is undoubtedly not unique for rabbits, since it has been shown that there is rapid blood-to-gut (both small and large bowel) lumen transport of sodium in dogs and blood to gastric juice transport of sodium in humans (18, 21). These data also indicate that estimates of total exchangeable sodium by radiosodium dilution include intraluminal gastrointestinal sodium, as is the case in other transcellular fluids, such as cerebrospinal fluid and pancreatic juice (21–23). Isotopic exchange, however, does not indicate the nature of the mechanism of gastrointestinal transport. It establishes that there is blood-to-gut transport but does not distinguish among passive diffusion, exchange diffusion, electroosmosis or active transport dependent on metabolic energy.

The amount of intraluminal gastrointestinal sodium in the rabbit is substantial. It is of interest that in this species, 14.2 per cent of the Na_e is in the gut, and 72 per cent of this quantity or 10.2 per cent of the Na_e is in the cecum and proximal half of the transverse colon. The distal colon contains only 1.9 per cent of the "total" gastrointestinal sodium, and the stool excretion rates averaged 2.9 per cent of the "total" gastrointestinal sodium or 0.4 per cent of the Na_e (*cf.* Table V). Visscher, Varco, Carr, Dean, and Erickson (18) found that sodium flux *out* of the colon exceeded sodium flux *into* the colon considerably in dogs. Judged by these findings the proximal and mid-colon conserve sodium quite efficiently. The human gastrointestinal tract may contain much less sodium than the rabbit's, since postmortem analyses gave values averaging 1.6 per cent of the predicted Na_e, and its distribution differs in that the small intestine contains 46 per cent of this quantity, with the remainder about equally distributed between the stomach and proximal colon (*cf.* Table VI). We cannot assume, of course, that these values apply to the living normal subject. Differences in gastrointestinal sodium content between rabbit and postmortem man may have significance, however, in view of similar differences in intraluminal gastrointestinal water contents between various species noted by Cizek (10). The collection of gastrointestinal fluid at postmortem after catastrophic illness, needless to say, does not afford a sound basis for estimating normal values. The justification for this examination rests in the fact that animal data cannot be transposed to humans. Indeed, premortem shifts of sodium under hormonal or other physiological influences may account for the low intraluminal sodium contents found in the human subjects.

The total exchangeable sodium of normal adult humans averages 41.0 mEq. per kgm. of body weight (12, 21, 22). The young adult rabbit has a mean Na_e of 46.0 mEq. per kgm. of body weight, which is significantly greater than the human value (*cf.* Table II). This difference may be due, at least in part, to greater intraluminal gastroin-

testinal sodium content in the rabbit. "Total" gut sodium averages 14.2 per cent of the Na_e, which is equivalent to 6.5 mEq. per kgm. of body weight (*cf.* Table IV). In the human subjects studied postmortem, "total" gastrointestinal sodium averaged 0.54 mEq. per kgm. of body weight. The additional 6.0 mEq. of sodium per kgm. of body weight in the rabbit gut would, therefore, neatly match the difference in the Na_e of 5.0 mEq. per kgm. of body weight between the two species. Since postmortem studies after catastrophic illness are obviously not adequate for judgment of normal values, there is a need for further studies in human subjects, particularly in previously well individuals soon after sudden accidental death.

Kruhøffer (24) estimated the interstitial fluid-plasma volume in rabbits by inulin volume of dilution to be slightly less than 20 per cent of the body weight. This volume would contain approximately 60 mEq. of sodium. If there is a shift of sodium from the gastrointestinal tract to the interstitial-plasma compartment in response to metabolic need, gut sodium could replace up to 20 per cent of the interstitial-plasma sodium in rabbits. During short fasting periods, however, we found little or no redistribution of sodium between gut and carcass (*cf.* Figure 2). In humans, shifts into this compartment might produce significant internal loss of sodium from the interstitial-plasma compartment. The finding of decreased fecal extraction of sodium with cation exchange resins in patients with edema suggests that disease states may alter sodium exchange or induce shifts of sodium across intestinal mucosa (25).

The equilibration of radiosodium with transcellular sodium is of significance in the interpretation of body sodium anatomy based on *simultaneous* dilution methods using inulin and sucrose. These saccharides apparently do not penetrate gastric juice, bile or cerebrospinal fluid (24, 26–28). The estimation of intracellular sodium by subtraction of the sodium in the inulin or sucrose volume of dilution from the total exchangeable sodium would therefore be in error in proportion to the quantity of transcellular sodium in the animals under study.

SUMMARY

Intraluminal gastrointestinal sodium content was measured in rabbits and in humans studied postmortem. In the former, gut sodium was referred to Na_e estimated with Na^{24}, while in the latter, gut sodium was referred to predicted Na_e values.

Total exchangeable sodium averaged 46.0 ± 2.0 mEq. per kgm. of body weight in rabbits. Of this, 14.2 ± 2.4 per cent was found to be in the gastrointestinal tract, with 0.9 ± 0.4 per cent in the stomach, 3.2 ± 1.2 per cent in the small intestine, and 10.2 ± 2.1 per cent in the proximal half of the large intestine. Radiosodium exchange equilibrium was virtually complete 24 hours after injection at all levels of the gastrointestinal tract. Fasting periods of 8 to 43 hours did not affect the quantity or distribution of gastrointestinal sodium to any appreciable extent. No significant difference in either Na_e or the quantity of intraluminal gastrointestinal sodium was found between male and female rabbits.

Human subjects at postmortem after a variety of diseases had relatively small amounts of intraluminal gastrointestinal sodium; the mean values were 1.6 per cent of the predicted Na_e in the "total" gastrointestinal tract, with 0.45 per cent in the stomach, 0.74 per cent in the small bowel and 0.36 per cent in the proximal portion of the large bowel. These data, however, cannot be taken as a reliable guide to the quantity of intraluminal sodium in living man.

The implications of these data are discussed in terms of the dynamics of sodium transport across the intestinal mucosa and the anatomy of body sodium.

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APPENDIX

Summary of case histories

F-1. Patient G. A., SFH 126096, female, aged 77 years. Patient expired 24 hours after admission.

Postmortem examination revealed: marked coronary atherosclerosis, myocardial fibrosis, recent infarct of the interventricular septum, 600 ml. of fluid in both pleural cavities, moderate pulmonary edema, calcification of the abdominal aorta and chronic cholecystitis with cholelithiasis.

M-2. Patient J. F., SFH 56451, male, aged 53 years. Patient expired in coma 11 days after admission.

Postmortem examination revealed: mitral stenosis, congestive heart failure with passive congestion of lungs, liver, spleen and kidneys, mural thrombus of left auricle, multiple pulmonary emboli, multiple emboli to brain stem, saddle embolus at bifurcation of the aorta, chronic pyelonephritis and chronic cholecystitis with cholelithiasis.

M-3. Patient J. W., SFH 179284, aged 51 years. Admitted in coma, with a known history of hypertension. Patient expired 1 hour after admission.

Postmortem examination revealed: hypertrophy of both ventricles, marked arteriolar nephrosclerosis, pulmonary congestion, occlusion of the right vertebral artery with multiple cerebellar and pontine infarcts, and coronary artery atherosclerosis with focal myocardial fibrosis.

F-4. Patient B. N., SFH 1693, female, aged 54 years. Present admission was for diabetic acidosis and coma. Patient expired 19 days after admission.

Postmortem examination revealed: necrotic pituitary tumor, type undetermined, mild glomerulosclerosis, bilateral cataracts, generalized arteriosclerosis involving the heart, kidneys and peripheral arteries, and a bilateral bronchopneumonia.

M-5. Patient F. D., SFH 133435, male, aged 64 years. Patient died 38 days after admission.

Postmortem examination revealed: pulmonary squamous cell carcinoma of left lower lobe with metastases to mediastinal and abdominal lymph nodes, pericardium and adrenals, acute bilateral bronchopneumonia, and generalized arteriosclerosis involving the aorta, coronary and renal arteries.

F-6. Patient N. R., SFH 134788, female, aged 56 years. Patient expired in coma 5 days after admission.

Postmortem examination revealed: cerebral atherosclerosis with encephalomalacia of right cerebral hemisphere, coronary atherosclerosis, left ventricular hypertrophy, nephrosclerosis, pulmonary emphysema and bronchopneumonia, moderate bronchiectasis, mild mitral stenosis, mild passive congestion of the liver, moderate fibrosis of the thyroid gland and chronic cholecystitis with cholelithiasis.

F-7. Patient G. W., SFH N-132431, female, aged 68 years. Patient expired two months after admission, soon after the onset of severe chest pain.

Postmortem examination revealed: healing myocardial infarction of the posterior left ventricle, chronic passive congestion of the lungs, liver and spleen, nephrosclerosis and chronic cholecystitis with cholelithiasis.

M-8. Patient G. H., SFH 135018, male, aged 69 years. Patient expired 11 days after admission.

Postmortem examination revealed: severe coronary atherosclerosis with an area of healed myocardial infarction, bilateral bronchopneumonia, demyelinization and gliosis of the basal ganglia, arteriosclerosis involving the aorta, renal and cerebral vessels, and chronic cholecystitis with cholelithiasis.

F-9. Patient A. F., SFH 135355, female, aged 67 years. Patient expired 43 days after admission.

Postmortem examination revealed: generalized arteriosclerosis with cerebral thrombosis and infarction, congenital atrophy of the right kidney, and marked bilateral pulmonary congestion.

M-10. Patient L. Y., SFH N-136139, male, aged 59 years. Patient expired five hours after admission.

Postmortem examination revealed: dissecting aneurysm of ascending aorta with hemopericardium, generalized arteriosclerosis and focal pulmonary fibrosis.

M-11. Patient I. P., SFH N-135687, male, aged 72 years. On the thirty-fourth hospital day patient developed severe pulmonary edema, became hypotensive and died a few hours later.

Postmortem examination revealed: coronary atherosclerosis with recent and old areas of myocardial infarction and a ventricular aneurysm, bilateral moderate nephrosclerosis, chronic passive congestion of the lungs, liver and spleen, and severe bilateral bronchopneumonia.

M-12. Patient A. D., SFH N-136536, male, aged 50 years. Patient expired on the sixth postoperative day following craniotomy for a pituitary tumor.

Postmortem examination revealed: cerebral infarction of left posterior hemisphere, a chromophobe adenoma of the pituitary and bilateral bronchopneumonia.

M-13. Patient A. T., SFH 17353, male, aged 76 years. Patient died suddenly 4 days after admission for post-traumatic quadriplegia.

Postmortem examination revealed: demyelinization of cervical spinal cord, generalized arteriosclerosis, and severe bilateral bronchopneumonia.

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GASTROINTESTINAL WATER AND ELECTROLYTES. II. THE EQUILIBRATION OF RADIOPOTASSIUM IN GASTROINTESTINAL CONTENTS AND THE PROPORTION OF EXCHANGEABLE POTASSIUM (K_e) IN THE GASTROINTESTINAL TRACT [1]

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Only 1.5 to 2.5 per cent of total body potassium is found in the extracellular fluid as estimated by tracer dilution methods (1–3). The usual methods for estimating extracellular potassium content, however, do not take into account fluid contained in the lumen of the gastrointestinal tract or in other transcellular fluid (4, 5).

The concentration of potassium in the secretions of the gastrointestinal tract is equal to or greater than that in plasma. In saliva, the concentration is approximately 4 times that of plasma, in gastric juice 2 to 3 times that of plasma, and in small and large intestinal secretions 1.5 to 2.5 times that of plasma (6–8). Abnormal loss of gastrointestinal fluids is known to produce rapid potassium depletion due to the loss of potassium contained in gastrointestinal secretions, as well as continued renal excretion of potassium (9, 10).

Endogenous potassium contained in the gastrointestinal tract is transported, at least in part, from blood to gut by secretory mechanisms. Using tracer techniques Corsa, Olney, Steenburg, Ball, and Moore (1) demonstrated that bile potassium is in equilibrium with the total pool of exchangeable potassium 10 hours after intravenous administration of radiopotassium. Exogenous potassium is rapidly absorbed from the gastrointestinal tract after the oral administration of isotopic potassium to rabbits; equilibrium between the intestinal contents and serum is almost complete in one hour (11). In view of these findings, an analysis of the equilibration of isotopic potassium in gut contents and estimation of the amount of potassium in the alimentary tract was considered worthwhile. The known role of gastrointestinal losses in the genesis of potassium depletion is an additional basis for this study.

The first paper in this series (4) reported the results of measurement of intraluminal gastrointestinal sodium content in rabbits and in human subjects studied postmortem. The present study is similar in design and execution to the study on gastrointestinal sodium content and presents observations on a) the amount of intraluminal gastrointestinal potassium relative to total exchangeable potassium (K_e) and the extent of radiopotassium exchange equilibrium in gastrointestinal contents in rabbits and b) the amount of intraluminal gastrointestinal potassium in human subjects studied postmortem.

METHODS

A. Rabbits

Forty-four adult albino rabbits were studied in pairs consisting of a male and a non-gravid female. Water was permitted *ad libitum* from the time of isotope injection until sacrifice, but food was withheld during this period.

Each animal was injected intraperitoneally with 250 to 350 microcuries of $K^{42/43}Cl$ [4] from calibrated syringes. The solution was made isotonic with NaCl, sterilized, and neutralized to pH 7. No contamination with other isotopes was present.

Urine, stool, gastrointestinal contents and blood were

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[4] The $K^{42/43}$ mixture was prepared in the Crocker Radiation Laboratory, University of California, Berkeley, by alpha particle bombardment of argon. At the time of radioassay the resultant mixture of K^{43} (half-life, 12.4 hours) and K^{42} (half-life, 22.4 hours) contained approximately 75 per cent K^{43} and 25 per cent K^{42}. K^{42} emits 0.81 and 0.24 Mev electrons and 0.4 Mev gamma-transitions.

collected for assay of radioactivity and chemical analyses as described in the first paper in this series (4). In some instances erythrocytes were aspirated from below the separated plasma of centrifuged heparinized blood. These red cells were then hemolyzed in 3 to 4 times their volume of distilled water. After being filtered through paper, aliquots were taken for assay of radioactivity and chemical analysis.

One-ml. aliquots of plasma, lyzed erythrocytes, bladder urine, gastrointestinal contents and excreted urine were plated in triplicate on filter paper, dried under an infrared lamp and covered with Parafilm. Three separate dilutions of the injected isotope solution were plated in triplicate and processed as above. Radioactive assay in the first 6 rabbits studied was carried out with an aluminum-covered end-window, thallium-activated sodium iodide scintillation counter and autoscaler. Because of the slow counting rates obtained with the scintillation counter, radioassays on samples obtained from the following 38 rabbits were measured with a thin mica end-window Geiger-Müller tube [5] and autoscaler. Preliminary observations revealed no significant self-absorption loss up to twice the quantity of solids present in the experimental samples. Correction for coincidence loss was unnecessary; with the counting tube used, no significant coincidence loss occurred at counting rates up to 4000 counts per minute. The counting rates did not exceed this value.

All samples were corrected for background radiation and radioactive decay. A separate decay curve was constructed for each shipment of $K^{42/43}$. Three separate dilutions of the isotope solution, each plated in triplicate, were assayed for radioactivity immediately before, at three intervals during, and immediately after the biological samples were assayed.

Triplicate potassium analyses were made from duplicate dilutions on aliquots of serum, lyzed erythrocytes, bladder urine, gastrointestinal contents, and excreted urine and stool with a lithium internal standard flame photometer.[6] The sodium concentration was similarly determined on these samples. A correction was made for the contribution of sodium emissions to the potassium assay when necessary.

B. *Human subjects*

Thirteen human subjects were studied at the time of postmortem examination. The age, sex, weight and height of each subject was recorded. Patients with clinically demonstrable edema or organic gastrointestinal disease were eliminated from the study. The gross and microscopic postmortem findings were subsequently recorded. The techniques employed in collecting and analyzing the gastrointestinal contents are described in the first report in this series (4); the appendix to the first report summarizes the pertinent features of the case histories of these subjects.

Triplicate potassium analyses were made from duplicate dilutions of the separate gastrointestinal contents using a lithium internal standard flame photometer.

[5] Radiobiological Apparatus Co., Berkeley, Calif.

[6] Baird Associates, Cambridge, Massachusetts.

CALCULATIONS

A. *Rabbits*

The total exchangeable potassium (K_e) was calculated from the dilution formula (1, 12):

$$K_e = \frac{K_i^{42/43} - K_o^{42/43}}{K_u^{42/43}/K_u^{39}} \qquad (1)$$

where:

$K_i^{42/43}$ = Injected radiopotassium in counts per minute.

$K_o^{42/43}$ = Radiopotassium excreted in the urine (bladder plus cage urine) to time of equilibration bladder urine sample.

$K_u^{42/43}/K_u^{39}$ = Specific activity of bladder urine at equilibrium of distribution in counts per mEq.

The degree of ionic exchange of intraluminal potassium with serum potassium was evaluated by relating gastrointestinal specific activities to the bladder urine specific activities.

$$\text{Specific activity ratio (S.A.R.)} = \frac{(S.A.)_t}{(S.A.)_u} \qquad (2)$$

where:

$(S.A.)_t = K_t^{42/43}/K_t^{39}$ = Tissue specific activity at equilibrium of distribution in counts per mEq.

$(S.A.)_u = K_u^{42/43}/K_u^{39}$ = Bladder urine specific activity at equilibrium of distribution in the counts per mEq.

The value of this ratio is assumed to represent the fraction of gut potassium which was exchanged (4, 13).

To determine the validity of using bladder urine specific activity as a standard of reference for the specific activity of the gastrointestinal contents, simultaneous plasma specific activity and bladder urine specific activity were determined in 14 cases 21 to 24 hours after the injection of radiopotassium (Table I). The mean ratio of specific

TABLE I

Plasma versus bladder urine specific activity in the rabbit 21 to 24 hours after the injection of radiopotassium

Rabbit	Specific activity plasma *cts./mEq.* $\times 10^3$	Specific activity bladder urine *cts./mEq.* $\times 10^3$	Specific activity bladder urine / Specific activity plasma
1	119.3	116.3	0.97
2	113.6	114.8	1.01
3	63.73	73.20	1.14
4	65.60	71.00	1.08
5	81.12	94.53	1.16
6	85.32	83.53	0.98
7	77.89	74.51	0.95
8	84.10	80.01	0.95
9	94.67	102.8	1.09
10	86.56	90.26	1.05
11	99.13	96.67	0.97
12	102.7	104.6	1.02
13	90.96	109.3	1.20
14	113.4	114.2	1.00
		Mean:	1.04
		Standard deviation:	±0.08

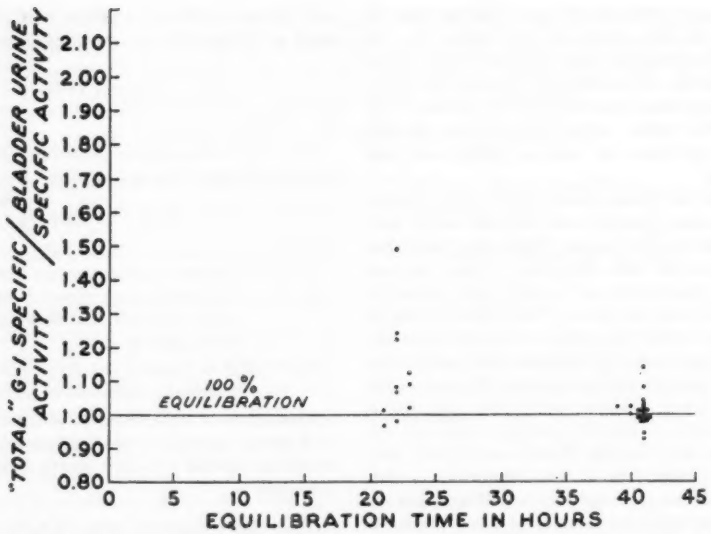

FIG. 1. THE EQUILIBRATION OF GASTROINTESTINAL POTASSIUM WITH CARCASS POTASSIUM, USING THE BLADDER URINE SPECIFIC ACTIVITY AS REPRESENTATIVE OF PLASMA SPECIFIC ACTIVITY, AS A FUNCTION OF THE TIME ALLOWED FOR ISOTOPE EQUILIBRATION

activity bladder urine/specific activity plasma was 1.04± 0.08. These results confirm those of Corsa and his colleagues (1). Because of errors which might be introduced by minimal hemolysis of blood in the preparation of plasma for assay and because of the relatively small amount of radioactivity in the plasma (due to the low potassium concentration), the specific activity of bladder urine was used as the more reliable standard of reference.

B. Human subjects

Samples from the human subjects were obtained and treated as described previously (4). The predicted K_e was estimated from published normal standards (14), taking into account the sex of the patient. Body weight was used as the reference unit. From studies on K_e in disease states, it is likely, however, that these estimates are generally too high up to a factor of 2 (14).

RESULTS

A. Rabbits

The effect of varying the time allowed for radiopotassium distribution on the ratio of total gastrointestinal to bladder urine specific activity is depicted in Figure 1.

A specific activity ratio of 1.00 is assumed to represent 100 per cent exchange. At 20 to 24 hours the specific activity ratio is quite variable,

TABLE II

The exchangeable potassium (K_e) and gastrointestinal potassium content of male versus female rabbits

	Male	Female	t	p
Number	13	13		
Body weight in kgm.±s.d.*	2.05±0.14	2.02± 0.13	0.55	>0.5
K_e in mEq.±s.d.	107.0 ±9.2	100.8 ±10.1	1.90	>0.1
K_e/body weight in mEq./kgm.±s.d.	52.2 ±4.3	49.8 ± 2.7	1.70	>0.1
"Total" G-I potassium† as % of K_e±s.d.	5.9 ±1.8	5.5 ± 2.4	0.422	>0.6

* s.d. = standard deviation = $\sqrt{\frac{\Sigma(x-\bar{x})^2}{n}}$.

† "Total" G-I potassium refers to the intraluminal potassium content of the gastrointestinal tract from the cardia of the stomach to the mid-transverse colon.

TABLE III

The exchangeable potassium (K_e) content in the rabbit *

	Body weight *kgm.*	Serum K *mEq./L.*	Exchangeable K *mEq.*	Exchangeable K *mEq./kgm.*
Number	32	32	32	
Mean	2.07	3.63	104.9	50.7
s.d.	±0.16	±1.51	±9.7	±4.0
Coefficient of variation	7.5%	41.6%	9.2%	8.0%

* K_e calculated 21 to 42 hours after the injection of radiopotassium.

often exceeding a value of 1.00 considerably. At 39 to 41 hours, however, the specific activity ratios cluster closely around 1.00, averaging 1.01, with a range of 0.93 to 1.14. It appears, therefore, that approximately 40 hours are required for uniform distribution of isotopic potassium between gut contents and the carcass.

Previous studies on normal human subjects have shown a sex difference in exchangeable potassium content; male subjects averaged 46.3 mEq. of potassium per kgm. of body weight, while female subjects averaged 39.0 mEq. per kgm. of body weight (14). To evaluate the possibility of similar sex differences in K_e of rabbits, pairs of animals, matched for weight, were studied; the data are presented in Table II.

The K_e averaged 6.2 mEq. greater in male rabbits than in female rabbits. The differences, however, in K_e, K_e per kgm. of body weight, and "total" gastrointestinal potassium expressed as per cent of the K_e were not statistically significant. Subsequent tabulations, therefore, were made without regard to the sex of the animal. In making this comparison, equilibration and fasting periods of 21 hours were employed for the most part. "Total" gastrointestinal potassium was distinctly lower in this group than in the animals subjected to 39 to 41-hour equilibration-fasting intervals (*cf.* Table VI), in spite of the fact that the difference in the mean K_e between these two groups of animals was not significant.

The exchangeable potassium content and the serum potassium concentrations are summarized in Table III. No significant difference in either K_e or K_e per kgm. of body weight was found between the animals allowed 21 hours of equilibration of radiopotassium and those allowed 40 hours. In 13 rabbits, in which equilibration-fasting periods of 21 to 24 hours were employed, the K_e averaged 52.3 ± 3.5 mEq. per kgm. of body weight, while the mean K_e in 23 rabbits after 39 to 42 hours of equilibration was 49.9 ± 3.3 mEq. per kgm. of body weight ($0.10 > p > 0.05$). The average K_e for the combined series was 104.9 mEq., or 50.7 mEq. per kgm. of body weight, with a coefficient of variation of 8.0 per cent (*cf.* Table III).

TABLE IV

The equilibration of $K^{42/43}$ with intraluminal gastrointestinal potassium in the rabbit *

	Number	Specific activity ratio† Mean	s.d.	t	p
Stomach	23	1.00	±0.043	0	1.0
Small intestine	23	1.01	±0.029	1.66	>0.1
Cecum and transverse colon	23	1.01	±0.068	0.704	>0.4
"Total" gastrointestinal tract	23	1.01	±0.042	1.14	>0.2

* $K^{42/43}Cl$ was injected 39 to 41 hours prior to sacrifice; the rabbits were fasted during this period.

† This ratio was obtained by dividing the specific activity of the gastrointestinal contents by the specific activity of the bladder urine.

The question of exchangeability of gut potassium at various levels of the gastrointestinal tract was explored by measuring the intraluminal specific activity relative to bladder urine specific activity. These data are summarized in Table IV. All of the animals in this group were studied 39 to 41 hours after the injection of radiopotassium. At all levels of the gastrointestinal tract the mean specific activity ratio did not differ significantly from a value of 1.00, being 1.00 ± 0.043, 1.01 ± 0.029, 1.01 ± 0.068, and 1.01 ± 0.042 for the stomach, small intestine, cecum and proximal transverse colon, and the "total" gastrointestinal

TABLE V

The specific activity of erythrocyte potassium relative to bladder urine specific activity with respect to the time allowed for equilibration of radiopotassium

Number	Equilibration time *hours*	R.B.C. specific activity / Bladder urine specific activity * Mean	Range
6	21–23	0.49	0.46–0.54
6	40–41	0.66	0.61–0.77

* A specific activity ratio of less than 1.0 indicates incomplete exchange equilibrium.

TABLE VI

The intraluminal gastrointestinal potassium content in the rabbit *

	Stomach		Small intestine		Cecum and transverse colon		"Total" G-I†	
	mEq.	*% of K_e*	*mEq.*	*% of K_e*	*mEq.*	*% of K_e*	*mEq.*	*% of K_e*
Number	21		21		21		21	
Mean	0.7	0.7	1.2	1.2	4.5	4.5	7.1	7.2
s.d.	±0.3	±0.3	±0.4	±0.4	±3.1	±2.7	±2.8	±2.2
Range	0.3–1.7	0.3–1.6	0.5–2.2	0.5–2.3	1.7–12.8	1.5–11.4	2.9–15.6	2.6–13.9

* Period of fasting and equilibration of isotope was 39 to 41 hours.
† "Total" G-I potassium refers to the intraluminal potassium content of the gastrointestinal tract from the cardia of the stomach to the mid-transverse colon.

tract, respectively. It is apparent, therefore, that intraluminal potassium is completely exchangeable with the body potassium pool.

Radiopotassium dilution, even after 40 hours of equilibration, however, does not measure true total body potassium content. Erythrocyte potassium exchange is still incomplete 40 hours after injection. Red cell specific activity relative to bladder urine specific activity is 0.49 and 0.66 at 21 to 23 and 40 to 41 hours of equilibration, respectively (*cf.* Table V). These findings agree closely with those of Corsa and his associates (1). Because of the short half-life of radiopotassium (K^{42} = 12.4 hours, K^{43} = 22.4 hours), it is impractical to extend the period of tracer equilibration beyond 40 hours. This technique does measure approximately 95 per cent of body potassium, which is sufficient for the purposes of this study (1).

The amount and distribution of intraluminal potassium is summarized in Table VI. "Total" gastrointestinal potassium averaged 7.2 ± 2.2 per cent of the K_e. The cecum and proximal half of the transverse colon contained 4.5 ± 2.7 per cent of the K_e, with lesser quantities in the small intestine and stomach, namely 1.2 ± 0.4 and 0.7 ± 0.3 per cent, respectively. The potassium content of the proximal portion of the large bowel accounts for some 63 per cent of the total gastrointestinal potassium pool. In a similar series of rabbits the bulk of the gut sodium pool (72 per cent) was also found in the proximal segment of the large intestine (4). The range of values for gut potassium was quite large in spite of the rigid fasting conditions imposed. In this study as little as 2.6 per cent of the K_e and as much as 13.9 per cent of the K_e was contained in the gut.

The distal colon contains very little potassium, some 2.8 per cent of the "total" intraluminal potassium, at the end of a 39 to 41-hour fasting period. Fecal excretion of potassium during a 39 to 41-hour fasting period was moderate in absolute magnitude, 2 mEq. per day (range, 0 to 5 mEq. per day), which is only 1.9 per cent of the mean K_e (*cf.* Table VII). In relation to the pool size of gastrointestinal potassium, however, the fecal excretion rate is quite significant, amounting to 33 per cent of the "total" intraluminal potassium per day. In contrast, the rate of stool sodium excretion averages 0.4 mEq. per day, which is only 2.6 per cent of the "total" intraluminal sodium content (4).

Fecal excretion of potassium in fed rabbits is of considerable absolute magnitude. Three rabbits allowed pellets [7] containing 53 mEq. of potassium per 100 grams, *ad libitum,* consumed 50 mEq. of potassium per day, or 50 per cent of their total exchangeable potassium content. In these animals fecal excretion rates varied from 4 to 13 mEq. of

TABLE VII

The potassium content of the distal colon and the rate of stool potassium excretion in the rabbit

	Descending colon and rectum		Stool potassium per 24 hours	
	Total mEq.	*% of "Total" G-I potassium**	*Total mEq.*	*% of "Total" G-I potassium**
Number	12		12	
Fasting period	39–45 hours		39–45 hours	
Mean	0.18	2.8	2.0	33
Range	0.04–0.41	0.6–5.0	0–5.0	0–87

* "Total" G-I potassium refers to the intraluminal potassium content of the gastrointestinal tract from the cardia of the stomach to the mid-transverse colon.

[7] Pillsbury's Best Foods, 16 per cent protein rabbit pellets.

TABLE VIII

Intraluminal gastrointestinal potassium content in the human studied postmortem

Sex	Pathological diagnosis	Post-mortem interval *hours*	Age *yrs.*	Body weight *kgm.*	Predicted K_e *mEq.*	Stomach			Small intestine			Cecum and proximal transverse colon			"Total" G-I		
						mEq.	*mEq./ kgm.*	*% of K_e*	*mEq.*	*mEq./ kgm.*	*% of K_e*	*mEq.*	*mEq./ kgm.*	*% of K_e*	*mEq.*	*mEq./ kgm.*	*% of K_e*
F-1	Recent myocardial infarction	6	77	35.0	1,425	1.2	0.03	0.08	6.7	0.19	0.47	1.5	0.04	0.10	9.3	0.27	0.66
M-2	Rheumatic heart disease with mitral stenosis	11	53	51.8	2,424	2.4	0.05	0.10	7.6	0.15	0.31	6.8	0.13	0.28	16.8	0.32	0.69
M-3	Hypertensive cardiovascular disease	22	51	43.6	2,040	6.0	0.14	0.29	8.2	0.19	0.40	2.4	0.06	0.12	16.6	0.38	0.81
F-4	Pituitary tumor	9	54	58.2	2,369	1.2	0.02	0.05	8.8	0.15	0.37	5.5	0.10	0.23	15.6	0.27	0.66
M-5	Squamous cell carcinoma of lung	7	64	43.6	1,775	5.7	0.13	0.32	7.4	0.17	0.42	13.4	0.31	0.76	26.6	0.61	1.50
F-6	Cerebral arteriosclerosis with encephalomalacia	6	56	58.2	2,369	2.3	0.04	0.10	4.3	0.07	0.18	18.7	0.32	0.79	25.2	0.43	1.07
F-7	Hypertensive and arterio-sclerotic heart disease	22	68	63.4	2,580	1.6	0.03	0.06	13.0	0.21	0.50	5.6	0.09	0.22	20.2	0.32	0.78
M-8	Paralysis agitans and bronchopneumonia	16	69	63.2	2,958	2.7	0.04	0.09	21.2	0.34	0.72	60.0	0.95	2.03	83.9	1.33	2.83
F-9	Cerebral thrombosis	20	67	65.0	2,646	7.7	0.12	0.29	8.4	0.13	0.32	0	0	0	16.1	0.25	0.61
M-10	Dissecting aneurysm of ascending aorta	19	59	68.0	3,182	1.0	0.02	0.03	16.1	0.24	0.51	1.3	0.02	0.04	18.4	0.27	0.58
M-11	Recurrent myocardial infarction	16	72	64.5	3,019	3.3	0.05	0.11	2.5	0.04	0.08	7.3	0.11	0.24	13.1	0.20	0.43
M-12	Chromophobe adenoma of pituitary	7	50	70.0	3,276	4.5	0.07	0.14	14.5	0.21	0.44	15.6	0.22	0.48	34.6	0.49	1.06
M-13	Traumatic demyelinization of cervical spinal cord	12	76	54.5	2,551	4.8	0.09	0.19	18.8	0.34	0.74	8.5	0.16	0.33	32.1	0.59	1.26
					Mean:	3.4	0.06	0.14	10.6	0.19	0.42	11.3	0.19	0.43	25.3	0.44	1.00
					Range:	1.0–	0.02–	0.03–	2.5–	0.04–	0.08–	0–	0–	0–	9.3–	0.20–	0.43–
						7.7	0.14	0.32	21.2	0.34	0.74	60.0	0.95	2.03	83.9	1.33	2.83

potassium per day, which is from 65 to 200 per cent of the "total" gut potassium content in the fasting state.

B. Human subjects

The results obtained on postmortem examination of human subjects are listed in Table VIII. The postmortem interval (the time between death and collection of gut contents) varied from 6 to 22 hours. No gross correlation between the quantity of gastrointestinal potassium and the postmortem interval is evident. Thirteen patients, 8 males and 5 females, whose ages at death ranged from 50 to 77 years, were studied. The clinical and pathological features of these cases are summarized in the appendix to the first report in this series (4). To express the intraluminal gastrointestinal potassium content in terms of the total labile pool of body potassium, a predicted K_e was calculated for each case, taking into account the patient's sex and body weight, from previously published normal values (14). Mean "total" gastrointestinal potassium was 0.44 mEq. per kgm. of body weight, or 1.0 per cent of the predicted K_e. The mean potassium content in the small intestine and proximal large intestine were equal, averaging 0.19 mEq. per kgm. of body weight, or 0.42 per cent of the predicted K_e; the stomach contents contained an average of 0.06 mEq. per kgm. of body weight, or 0.14 per cent of the predicted K_e. In man studied postmortem the distribution of gut potassium differs from the distribution of gut sodium, in that the cecum and proximal transverse colon contain about 43 per cent of gut potassium as against 22 per cent of gut sodium, while the stomach contains 14 per cent of gut potassium compared to 28 per cent of gut sodium (4).

DISCUSSION

The concentration of potassium in the gastrointestinal contents is almost always greater than that in plasma. In humans, the highest concentrations are found in saliva and small intestinal secretions, averaging 20 mEq. per liter and 11 mEq. per liter, respectively (6–8). The lowest concentrations are found in the biliary and pancreatic secretions, where they approximate that in plasma. Nevertheless, intraluminal gastrointestinal potassium has been largely ignored in past considerations of the distribution of potassium in the body.

Gastrointestinal potassium is obviously extracellular and is freely exchangeable with plasma potassium. The high concentrations of potassium in most gastrointestinal secretions are probably indicative of an active transport mechanism from blood to gut (15). Intestinal transport of both sodium and potassium are affected by adrenal hormones. ACTH-treated nephrectomized rats, both fed and fasted, have an increased gut potassium content; conversely, adrenalectomy decreases the potassium content of the gut in rats (16). Adrenalectomy in dogs produces a sharp decrease in the rate of intestinal absorption of potassium, as well as of sodium and chloride, which is rectified by the administration of adrenal cortical hormones (17). It is likely, therefore, that both blood-to-gut and gut-to-blood transport of potassium involves active mechanisms dependent on energy-yielding metabolic processes.

The gastrointestinal specific activity ratios of 1.00, 1.01, 1.01, and 1.01 for stomach, small intestine, proximal large bowel, and "total" gastrointestinal contents, respectively, demonstrate the complete exchangeability of gastrointestinal potassium (*cf.* Table IV). Gastrointestinal potassium, is, therefore, an integral part of the total body pool of labile potassium, and is measured when the total exchangeable potassium is determined by the isotope dilution technique.

The amount of potassium contained in the gastrointestinal tract of the rabbit greatly exceeds that found in the remainder of the extracellular fluid. Kruhøffer (18) estimated the volume distribution of inulin in rabbits to be almost 20 per cent of the body weight. This volume would contain approximately 1.6 mEq. of potassium in a rabbit weighing 2 kgm. The "total" gastrointestinal potassium content of rabbits fasting 39 to 41 hours averaged 7.1 mEq., which is almost four and one-half times as much as in the interstitial-plasma compartment. Gut potassium could completely replace the interstitial-plasma potassium in rabbits in response to a metabolic need or contribute considerably to abnormal elevations in serum potassium concentrations in pathological states. Swan and Pitts (19)

found that infusing acid loads in nephrectomized dogs resulted in a redistribution of body potassium and calculated a net increase of 31 mEq. of potassium in the interstitial-plasma fluid. It is conceivable that migration of potassium from gut to blood could account for a significant part of the increase in interstitial-plasma fluid potassium noted under these conditions.

The pool size of gut potassium may be considerably smaller in man than in rabbits. A 70-kgm. man has approximately 50 mEq. of potassium in the plasma-interstitial fluid compartment as estimated by thiosulfate dilution (20). In the postmortem studies on human subjects the intraluminal potassium content averaged 25 mEq., or approximately 50 per cent of the remainder of extracellular potassium (*cf.* Table VIII). These data obviously are not reliable, however, in making inferences relative to normal living subjects, since agonal influences may have induced marked shifts of potassium across the gut wall.

The mean K_e of young adult rabbits was 50.7 mEq. of body weight in this study (Table III). This value differs significantly from the mean of 56.1 mEq. of potassium per kgm. of body weight in rabbits reported by Corsa and his colleagues (1). The discrepancy between these two values in all probability is due to the differences in weight and age of the animals comprising each series. In this study the mean weight was 2.07 kgm., while in the study of Corsa and his colleagues (1) the mean weight was 1.06 kgm. Forbes and Perley (21, 22) have shown similar differences in exchangeable sodium content (*i.e.*, decreasing Na_e per kgm. of body weight) between children and adult humans. The animals in our series were 150 to 170 days old, while those reported by Corsa and his colleagues (1) were approximately 60 days old. Direct chemical analyses on sacrificed normal rabbits revealed a constant potassium content but an increase in body fat from approximately 4 per cent to 8 per cent of the body weight during this interval (23).

The total exchangeable potassium content is measurably lower in adult man than in the rabbit, averaging 43 mEq. per kgm. of body weight (46.3 mEq. per kgm. in males and 39.0 mEq. per kgm. in females) (14). This species difference may result, at least in part, from differences in intraluminal gastrointestinal potassium content. The estimated gut potassium content is 0.44 mEq. per kgm. of body weight in man at postmortem (Table VIII) and 3.5 mEq. per kgm. of body weight in rabbits. In other words, about half the difference in K_e may be accounted for on the basis of the greater content of intraluminal potassium in rabbits. Intraluminal potassium content in living man may differ considerably, however, from the quantity found at postmortem.

Gastrointestinal potassium amounts to 7.2 per cent of the K_e in rabbits, with 63 per cent of the "total" intraluminal potassium, or 4.5 per cent of the K_e, in the cecum and proximal half of the transverse colon (Table VI). This is similar to the intraluminal distribution of sodium, since 72 per cent of gut sodium is contained in the proximal portion of the large bowel (4). The distal colon, in contrast, was found to contain only 0.2 per cent of the K_e, or 2.8 per cent of "total" gastrointestinal potassium. The rate of fecal excretion of potassium in the fasting rabbit averages 2 per cent of the K_e, or 33 per cent of "total" gastrointestinal potassium per day (Table VII). The rate of fecal excretion of potassium is about five times the rate of fecal sodium loss (4). The conservation of potassium by the proximal large bowel in the fasting rabbit is considerably less efficient, therefore, than the conservation of sodium.

In a group of 13 human subjects studied postmortem after suffering from a variety of illnesses, the "total" gastrointestinal tract contained an average of 1.0 per cent of the K_e, with 0.14 per cent, 0.42 per cent, and 0.43 per cent in the stomach, small intestine and proximal colon, respectively (Table VIII). The variation in potassium content of the gut is considerable, however, ranging from as little as 9 mEq. to as much as 84 mEq. in the cases studied. The distal colon may contain or transport quite large amounts of potassium as judged by analyses of enema returns. Dunning and Plum (24) found as much as 250 mEq. of potassium in a single tap water enema efflux in a patient with neurogenic bowel dysfunction.

Methods for *in vivo* estimation of intracellular potassium are necessarily indirect. Such estimates, based on multiple tracer techniques when inulin or sucrose is used to measure extracellular fluid content, will be in error in proportion to the

amount of potassium in the transcellular fluid of the animal under study (4). Estimations of the concentration of potassium in intracellular water by these methods will be too low, since the ratio of potassium to water in transcellular fluid is small compared to the ratio in intracellular fluid (2, 5–8, 25).

SUMMARY

Intraluminal gastrointestinal potassium content was measured in rabbits and in human subjects studied postmortem. In rabbits gut potassium was referred to the K_e, as determined with $K^{42/43}$. In human subjects gut potassium was referred to predicted K_e values.

The total exchangeable potassium in rabbits averaged 50.7 ± 4.0 mEq. per kgm. of body weight. Of this, an average of 7.2 ± 2.2 per cent was contained in the "total" gastrointestinal tract, with 0.7 ± 0.3 per cent in the stomach, 1.2 ± 0.4 per cent in the small intestine, and 4.5 ± 2.7 per cent in the proximal half of the large intestine. Radiopotassium equilibrium was virtually complete at 40 hours after injection at all levels of the gastrointestinal tract, but not 24 hours after injection. No significant difference in K_e or the quantity of intraluminal gastrointestinal potassium was found between sexes.

Human subjects at postmortem examination had relatively small amounts of intraluminal gastrointestinal potassium; the mean values were 1.0 per cent of the predicted K_e in the "total" gastrointestinal tract, with 0.14 per cent in the stomach, 0.42 per cent in the small bowel, and 0.43 per cent in the proximal half of the large bowel.

It is estimated that in the rabbit approximately 80 per cent of the total extracellular potassium is located in the gastrointestinal tract. In man, it is estimated that one-third of the total extracellular potassium is located in the lumen of the gastrointestinal tract postmortem. The relative quantity of intraluminal potassium in the normal living subject cannot be reliably inferred from these data, however.

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THE CAUSAL ROLE OF PLASMA ALBUMIN DEFICIENCY IN EXPERIMENTAL NEPHROTIC HYPERLIPEMIA AND HYPERCHOLESTEREMIA [1]

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Previous studies (1–8) from this laboratory have indicated that the hypercholesteremia and hyperlipemia occurring in the experimental nephrotic syndrome induced in rats by injection of anti-rat kidney serum are due primarily to some intravascular "trapping" phenomenon whereby the animal appears unable to remove either endogenously-produced or dietary-derived cholesterol and lipid from the plasma with its customary efficiency. However, since renal changes seemingly initiate the total nephrotic syndrome, it seems probable that this "trapping" of cholesterol and lipid which occurs in the plasma in this syndrome is related in some manner to such renal changes. In the present study, an attempt was made to determine the nature of this relationship. The observations herein reported suggest (a) that the hypercholesteremia and the hyperlipemia observed in the experimental nephrotic syndrome are initiated and maintained by the external renal loss of some plasma constituent, and (b) that this substance specifically is albumin.

I. *The Relationship of Nephrotic Hyperlipemia and Hypercholesteremia to Renal Damage and External Renal Excretion*

Methods

Drastic alteration in renal function was accomplished in several series of experimental and control male rats (Long-Evans strain) by 1) nephrectomy, 2) bilateral ligation of renal pedicles, 3) bilateral ureteral ligation, and 4) ureter-vena caval anastomosis.

1) *Control rats.* Pooled rabbit anti-rat kidney serum (AKS) was prepared as described by Heymann and Lund (9). The hypercholesteremic effect of the AKS was established by injection of 1.0 ml. of it intravenously into 25 intact rats (group 1) and into 16 sham-operated (splenectomy) rats (group 2) that were bled 24 hours later for determination of plasma total cholesterol (10) and lipids (11). At the same time, 15 other normal control rats (group 3) that had been injected with 1.0 ml. of *normal* rabbit serum were bled in order to establish normal plasma lipid levels.

2) *Nephrectomy.* The second series of 45 rats was subjected to bilateral nephrectomy. Twenty-nine of these rats (group 4) were given intravenously 1.0 ml. of potent AKS immediately after the nephrectomy. The remaining 16 nephrectomized rats (group 5) served as controls, receiving 1.0 ml. of normal rabbit serum. Blood samples taken 24 hours later were analyzed for total cholesterol and those of group 4 also for total lipids. In addition, five other rats (group 6) that had been fed a protein-electrolyte-free diet (12), enabling them readily to survive for at least five days after nephrectomy, also were given 1.0 ml. of AKS immediately after nephrectomy.

3) *Bilateral ligation of the renal pedicles.* A third series of rats (group 7) was subjected to bilateral ligation of the renal pedicles by a method which obviated the possible acute effects of anesthesia and laparotomy on the animals' response to a hypercholesteremic excitant. This method consisted of the application of silk ligatures applied loosely around both renal pedicles. The two ends of the ligatures were exteriorized through incisions made in the opposite lateral abdominal walls and secured to the skin surfaces. The rats then were allowed to recover for 48 hours postoperatively, at which time the exteriorized ends of the ligatures were pulled taut, thus completely interrupting the blood supply to the kidney. At the completion of the experiment, all animals were autopsied and data were used only from those animals in which complete interruption of renal circulation was observed to have occurred. Eleven rats were ligated successfully in this fashion and immediately after were injected intravenously with 1.0 ml. of AKS. Blood samples obtained 24 hours after ligation were analyzed for total cholesterol and lipids.

4) *Bilateral ureteral ligation.* The fourth series of 37 rats was subjected to bilateral ureteral ligation by exactly the same method described above except that a loose ligature was applied about the ureter of each kidney and then tightened 48 hours later. Twenty-four of these rats (group 8) were given intravenously 1.0 ml. of AKS immediately following ligation and the remaining 13 rats

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(group 9) served as controls, receiving 1.0 ml. of normal rabbit serum. For additional control purposes, eight rats (group 10) that had been placed four days previously upon the uremia-retarding, protein and electrolyte-free diet were similarly ligated and injected with 1.0 ml. of AKS.

All rats were sacrificed 24 hours after injection of AKS, at which time an antemortem laparotomy was performed and examination made to ensure effective ureteral ligation. In order to test the effect of the latter procedure upon the renal circulation, three rats of each group as well as five other normal rats were injected intravenously with 1.0 ml. of 0.1 per cent trypan blue and their renal pedicles clamped one minute later. The kidneys then were removed, examined grossly for their content of dye under the dissecting microscope and sections were embedded in paraffin blocks but left unstained. In addition, sections of kidneys were obtained from five other rats of each group for staining with hematoxylin and eosin, Hotchkiss' periodic acid-Schiff's reaction and the Rinehart and Abul-Haj's modified Hale stain, as described by Weinreb, Soules, and Wissler (13). At the time of sacrifice all rats were bled for determination of total cholesterol and those of group 8 also for total lipids.

5) *Ureter-vena caval anastomosis.* The effect of preventing *external* loss of urine was studied further by devising an operation which although allowing glomerular filtration, nevertheless prevented external urine loss by its diversion into the inferior vena cava.

Under ether anesthesia, the abdomen of the rat was incised, the right ureter was doubly ligated and severed about 12 mm. from the renal pelvis. The proximal ligature, attached to a straight needle, was introduced via the right inferior lumbar vein into the inferior vena cava. The wall of the inferior vena cava opposite and slightly below the entrance of the lumbar vein was pierced with the needle and the ligature thus was pulled through this puncture until a small portion of its attached ureter also was withdrawn from the lumen of the inferior vena cava. The attachment of the ligature was then cut and the now patent distal end of the ureter was retracted back into the inferior vena cava to lie free to discharge urine therein. Simple pressure successfully prevented all bleeding from both the inferior lumbar vein and the vena cava. After this was done a loose ligature was placed around the pedicle of the left kidney and its ends brought out and secured to the skin surface as described previously. Three days later, under light ether anesthesia, the latter ligature was tightened to occlude completely the vascular pedicle of the left kidney (confirmed by subsequent autopsy).

Seven anuric rats (group 11) of this kind were obtained and injected with 1.0 ml. of AKS immediately after the ligation of the left renal pedicle. This last procedure of course produced a rat having its one functioning kidney emptying its urine into the venous circulation. For control purposes, a loose ligature was placed about only the left renal pedicle of three otherwise normal rats (group 12) and 72 hours later, the ligatures were tightened and the rats then were injected with 1.0 ml. of AKS. Blood samples obtained 24 hours after the injection of AKS were analyzed for total cholesterol. Just before sacrifice, each animal was injected with trypan blue and sections of the right kidneys of all rats were obtained and stained, as described above.

Results

A. *Control rats*

As shown in Table I the injection of anti-kidney serum into the control rats induced a rapid rise in plasma cholesterol, an average of 154 mg. per 100 ml. being observed 24 hours later in the intact rats (group 1) and 126 mg. per 100 ml. in the sham-operated rats (group 2), in contrast to the average plasma cholesterol of 64 mg. per 100 ml. in the control rats injected with normal rabbit serum (group 3). A parallel rise of total lipids occurred in the animals given anti-kidney serum.

B. *Effect of removal of kidneys upon hypercholesteremic effect of AKS*

As Table I indicates, the removal of both kidneys prevented the hypercholesteremic effect of injected AKS. Thus the degree of hypercholesteremia (82 mg. per 100 ml.) in the nephrectomized rats injected with AKS (group 4) was essentially the same as that in the control nephrectomized rats given normal rabbit serum (group 5) and considerably below that found in the intact (group 1) or sham-operated control rats (group 2). The possible debility of these uremic rats moreover was not responsible for their failure to respond to the hypercholesteremic effect of AKS since similar negative results were observed in the nephrectomized rats maintained upon the uremia-retarding diet (group 6).

Functional nephrectomy, achieved by bilateral ligation of the renal vascular pedicles (group 7), likewise prevented the usual hypercholesteremic response to injection of AKS. The bilateral nephrectomy or renal vascular pedicle ligation similarly prevented the rise of total lipids in the plasma which occurred in the control nephrotic rats (groups 1 and 2).

C. *Effect of prevention of external urine excretion upon hypercholesteremic effect of AKS*

1) *Bilateral ureteral ligation.* The injection of AKS into rats in whom external renal excretion

TABLE I

Plasma lipids in rats injected with rabbit anti-kidney serum following various procedures

Series	Group	No. of rats	Aver. wt. (gm.)	Plasma concentrations (24 hr. after injection of rabbit serum) Total lipids (mg./100 ml.)	Total cholesterol (mg./100 ml.)
1)	Control rats				
	1. Intact-injected-AKS*	25	184	373(309–476)‡	154(103–246)±6.6§
	2. Sham-operated-injected-AKS	16	166	435(304–676)	126(96–171)±5.6
	3. Intact-injected-NRS†	15	195	215(120–320)	64(48–73)±1.3
2)	Bilateral nephrectomy				
	4. Injected-AKS	29	209	180(133–250)	82(55–103)±2.7§
	5. Injected-NRS	16	185		90(72–115)±2.8
	6. Injected-AKS (special diet)	5	160		92(80–131)±2.8
3)	Bilateral ligation of renal pedicles				
	7. Injected-AKS	11	175	247(132–298)	98(77–113)±4.0
4)	Bilateral ureteral ligation				
	8. Injected-AKS	24	166	280(193–365)	87(64–110)±2.4
	9. Injected-NRS	13	178		98(71–114)±3.6
	10. Injected-AKS (special diet)	8	150		67(48–76)±1.2
5)	Unilateral ureter-vena caval anastomosis (left renal pedicle ligated)				
	11. Right ureter anastomosed: injected-AKS	7	236		88(78–107)±2.6
	12. Control-right ureter intact: injected-AKS	3	220		144(113–196)±21.4

* AKS = Rabbit anti-rat kidney serum.
† NRS = Normal rabbit serum.
‡ Figures in parentheses refer to range of values.
§ Standard error of the mean.

was completely abolished by bilateral ureteral ligation (group 8) did not lead to a greater degree of hypercholesteremia than that shown by the control rats, similarly operated, but injected with normal rabbit serum (group 9). Again the slight rise in cholesterol that was observed was that expected in the uremic animal (14) and again was considerably below that observed in both the intact (group 1) and sham-operated controls (group 2). Here again, as Table I indicates, even less of a rise in plasma cholesterol was observed in the ureter-ligated rats maintained on the special diet (group 10), ensuring a relatively good condition during the experimental interval.

The kidneys of the ureteral-ligated rats (groups 8, 9, 10) that were injected with trypan blue exhibited both grossly and microscopically almost as intense staining as that observed in the kidneys of the five normal rats similarly injected. Although this suggested that the integrity of the renal circulation was preserved during the 24-hour experimental interval, it seems likely that ligation of the ureters did affect the intrarenal circulation to some extent. On the other hand, it is unlikely that the latter would in the present instance affect the development of AKS-induced renal changes since AKS-antibodies are deposited in the kidney with great rapidity after their injection. That such is the case was suggested by comparison of the stained sections of the ureteral-ligated rats with those of the control rats (group 12) also injected with AKS 24 hours prior to study. Although neither the experimental nor the control rats exhibited the marked glomerular capillary basement membrane thickening and accumulation of acid mucopolysaccharide between the glomerular loops which are found at a later interval after injection of AKS (9, 13), both the experimental and control rats exhibited characteristic early glomerular changes which included a neutrophilic infiltrate, some loss of the delicate markings of the capillary tufts, and some accumulation of iron-positive mucoid material between the capillary loops. Both groups of rats also exhibited typical tubular changes, especially of the proximal convoluted tubules, including granularity, paleness and decreased staining of the epithelium (9, 13). Necrosis of glomeruli or of tubular cells was not observed in any rats.

2) *Ureter-vena caval anastomosis.* Similar

cholesterol and lipid results were obtained in the uninephrectomized rats whose remaining ureters emptied their contents back into the venous circulation (group 11). Thus, despite the fact that blood was circulating in the kidney and the glomeruli were functioning (see below), their hypercholesteremic response to AKS was the expected one from uremia alone (Compare with groups 5 and 9) and far below that observed in the control rats (group 12) which excreted urine in the usual fashion.

Similar to the findings in the kidneys of the ureteral-ligated rats, the kidneys of the rats with the ureter-vena caval anastomosis exhibited almost as intense staining with trypan blue as that observed in the kidneys of the control rats. Again early glomerular changes, similar to those described above, were observed in both groups (Figure 1). The tubular changes which included the presence of considerable amounts of excreted protein material in most of the tubules, appeared to be comparable in both the experimental and control groups, as shown in Figure 2. The finding of "proteinuria" demonstrates the altered glomerular function induced by AKS in both groups of rats.

II. *The Role of Plasma Albumin Deficiency in Nephrotic Hyperlipemia and Hypercholesteremia*

The previous studies suggested that the *external* loss of some plasma substance(s) via the urine of the rat, following injection of AKS, was the *sine qua non* for the development of the hypercholesteremia and hyperlipemia of the experimental nephrotic syndrome. Since albumin is one of the plasma substances known to be excreted in great quantity in this syndrome, studies were pursued to determine if the external loss of this substance was responsible for the development of this hyperlipemia and hypercholesteremia.

METHODS AND RESULTS

A. *The effect of anti-kidney serum injection on plasma protein concentrations*

Methods. Twelve male, Long-Evans rats (weight: 150 to 172 gm.) were bled from the tail one week prior to injection of 1 ml. of potent AKS and all rats again were bled 24 hours following this injection. Each sample of blood plasma was analyzed for total cholesterol and total lipids, and for total protein and albumin concentrations (15). In addition, urine was collected from 5 of the rats during a 24-hour interval prior to and again following injection of AKS, and analyzed for total protein content (16).

Results. The results are shown in Table II in which it can be seen that injection of AKS induced marked renal loss of protein sufficient to lower markedly plasma total protein and albumin concentrations within a 24-hour interval following injection of the immune serum. A concomitant rapid rise of total lipids and cholesterol was observed.

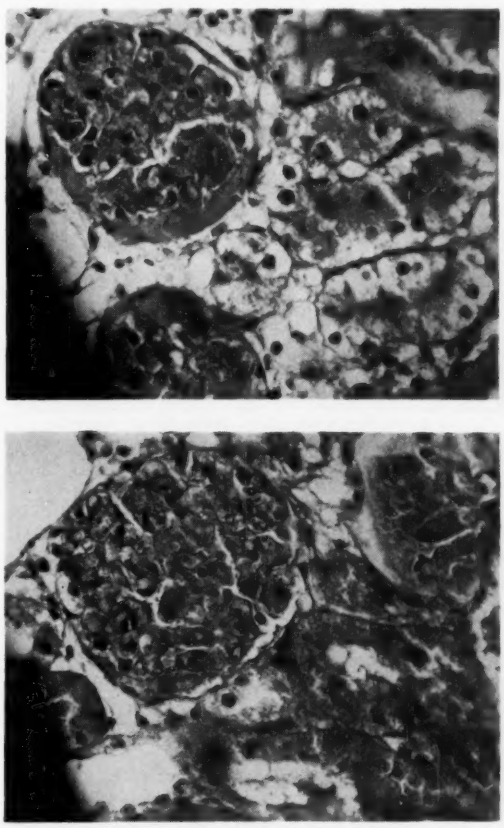

FIG. 1. (MODIFIED HALE STAIN × 400.) GLOMERULI OF RAT WITH URETER-VENA CAVAL ANASTOMOSIS 24 HOURS AFTER INJECTION OF AKS, SHOWING ENLARGED GLOMERULI AND INCREASE OF IRON-POSITIVE MATERIAL BETWEEN THE GLOMERULAR LOOPS

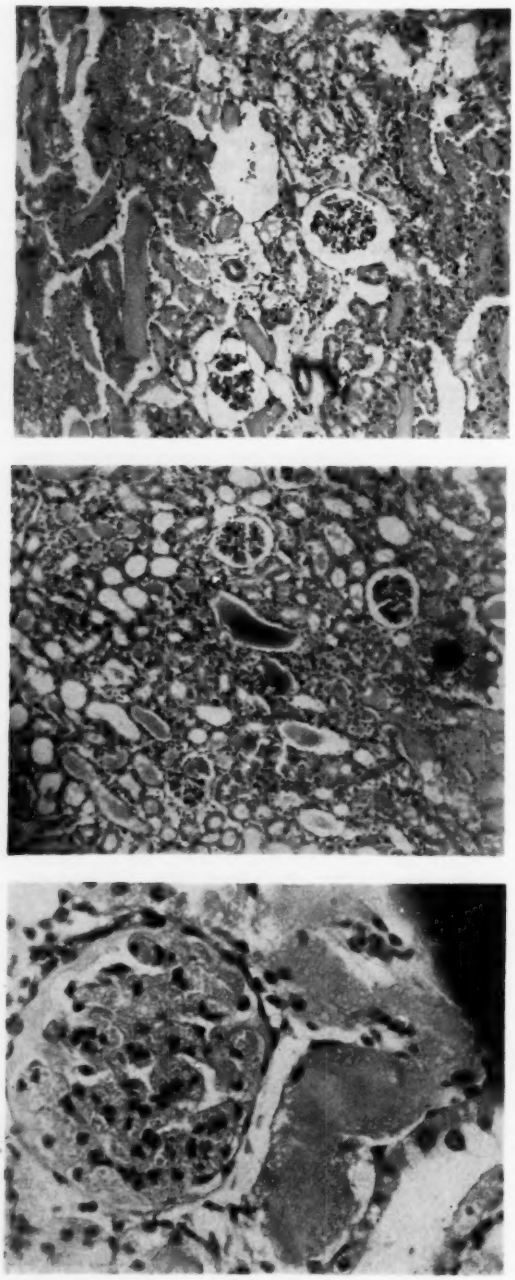

FIG. 2. (HEMATOXYLIN AND EOSIN.) TOP × 100—LOW POWER VIEW SHOWING PROTEIN IN RENAL TUBULES OF CONTROL RAT 24 HOURS AFTER AKS INJECTION. MIDDLE × 100 AND BOTTOM × 400—LOW AND MEDIUM POWER VIEWS OF RAT WITH URETER-VENA CAVAL ANASTOMOSIS 24 HOURS AFTER AKS INJECTION, SHOWING PROTEIN IN RENAL TUBULES, GLOMERULAR CHANGES AND GRANULARITY, SWELLING AND PALE STAINING OF TUBULES

TABLE II

Plasma protein and lipid changes induced by injection of anti-kidney serum *

	Prior to AKS injection	24 hours after AKS injection
Average plasma concentrations		
Total cholesterol (*mg./100 ml.*)	48 (39–63)†	153 (103–243)
Total lipids (*mg./100 ml.*)	183 (135–227)	407 (230–625)
Total protein (*gm./100 ml.*)	5.9 (5.4–6.3)	3.7 (3.4–4.8)
Total albumin (*gm./100 ml.*)	2.34 (2.03–2.63)	0.86 (0.43–1.44)
Average urine content		
Total protein (*mg./24 hr.*)	21‡ (16–30)	359‡ (135–585)

* Twelve rats.
† Range of values.
‡ Five rats only.

B. *The effect of albumin infusion on plasma proteins and lipids of rats injected with anti-kidney serum*

Methods. Each of a series of 40 rats was subjected to abdominal laparotomy under ether anesthesia during which an inferior lumbar vein was cannulated with a polyethylene cannula which was exteriorized through a stab wound. Following closure of the incision the rats were individually caged in restraining units and the intravenous cannulae were connected to a special infusion apparatus designed to inject 0.3 ml. of solution per hour continuously during a 24-hour interval. Each rat then was injected with one ml. of potent AKS. During the ensuing 24-hour interval 24 of the rats were each continuously injected with a 20 per cent aqueous solution of bovine serum albumin and the remaining 16 control animals were similarly infused, but with 0.45 per cent sodium chloride solution. The latter was used in the control rats because its retention in nephrotic plasma would probably induce some degree of hemodilution approximating that induced by infusion of albumin. Approximately 7.0 ml. of fluid thus was injected into each rat over the 24-hour period. All rats then were bled for determination of total lipids, total cholesterol and total protein and albumin. Hematocrit determinations also were made in 12 rats of each group in order to evaluate any difference in hemodilution.

In order to prevent the renal loss of the infused albumin solution, another group of rats was nephrectomized prior to the infusion. Thus a group of 10 rats was in-

TABLE III

Effect of albumin infusion on plasma proteins and lipids of intact and nephrectomized rats injected with anti-kidney serum

Infusion fluid	No. of rats	Aver. wt. (gm.)	Average plasma concentrations: Pre-nephrectomy Total cholesterol (mg./100 ml.)	Following 24-hour infusion: Total cholesterol (mg./100 ml.)	Total lipids (mg./100 ml.)	Total protein (gm./100 ml.)	Albumin (gm./100 ml.)
I. Rats injected with AKS prior to infusion							
1. Rats with intact kidneys							
Albumin	24	148		101 (46–145)*	282 (150–349)	6.79 (4.82–7.98)	4.67 (3.13–8.20)
Saline	16	150		169 (87–277)	366 (233–427)	4.78 (4.08–5.68)	1.85 (0.90–2.80)
2. Nephrectomized rats							
Albumin	5	164	85 (65–115)	49 (44–63)	202 (113–352)		7.37 (6.86–8.66)
Saline	5	172	72 (53–91)	107 (97–121)	263 (198–292)		2.11 (1.93–2.27)
II. Normal control rats							
None	12	160		48 (39–63)	183 (135–227)	5.90 (5.40–6.30)	2.34 (2.03–2.63)

* Range of values.

jected with the AKS and subjected to bilateral nephrectomy 16 hours later. During the ensuing 24-hour postoperative interval 5 of the rats were infused with the albumin solution and 5 with saline, as described above. All rats were bled for determination of total cholesterol at the time of nephrectomy and again for total lipids, total cholesterol and albumin following the infusion interval.

Results. The results are shown in Table III. The plasma concentrations obtained from 12 normal rats (from Table II) also are included for purposes of comparison. The control rats that were infused with saline for 24 hours following injection of AKS exhibited a fall of total protein and albumin and a rise of total lipids and cholesterol in their plasma similar to that observed in the untreated nephrotic rats (see Table II). In contrast, as also shown in Table III, the infusion of albumin for 24 hours following injection of AKS prevented the usual fall of plasma total proteins and albumin in this group of rats, and their rise of plasma total lipids and cholesterol was of considerably lesser magnitude in comparison to the control animals and entirely prevented in a number of these rats. No outward evidence of toxicity was apparent in either group of rats. The observed differences were not found due to significantly different states of hemodilution since the average hematocrit in 12 rats infused with albumin solution was 42 (Range: 35 to 50) while in the control rats infused with saline the average hematocrit was 50 (Range: 44 to 56).

At the time of nephrectomy, performed 16 hours after injection of the AKS, only a moderate rise of the average plasma cholesterol had occurred.

TABLE IV

Effect of albumin infusion on plasma lipids and proteins of rats with previously established nephrotic hypercholesteremia

Infusion fluid	No. of rats	Aver. wt. (gm.)	Average plasma concentrations: Prior to infusion Total cholesterol (mg./100 ml.)	Following 24-hour infusion: Total cholesterol (mg./100 ml.)	Total albumin (gm./100 ml.)
Albumin	8	152	239 (124–325)*	159 (54–286)	3.38 (2.27–4.35)
Saline	6	148	220 (117–325)	226 (135–336)	1.37 (1.05–2.77)

* Range of values.

TABLE V

Changes in plasma lipids and albumin following completion of albumin infusion in rats injected with AKS

Type of infusion	No. of rats	Aver. wt. (gm.)	Average plasma concentrations: Prior to infusion — Total cholesterol* (mg./100 ml.)	Following infusion: Immediately after infusion — Total cholesterol (mg./100 ml.)	24 hours after infusion terminated — Total cholesterol (mg./100 ml.)	Total lipids (mg./100 ml.)	Total albumin (gm./100 ml.)
Albumin	5	172	97 (41–133)†	90 (51–120)	194 (79–279)	823 (194–1,360)	1.58 (1.03–2.29)
Saline	5	168	99 (44–188)	167 (87–277)	221 (109–336)	920 (375–1,970)	1.00 (0.74–1.57)

* Sixteen hours after AKS injection.
† Range of values.

Removal of the kidneys prevented further renal loss of albumin and, as shown in Table III, albumin infusion into the nephrectomized rats previously injected with AKS was associated with a fall of their plasma cholesterol to normal levels. The concentration of plasma lipids and cholesterol in the saline-injected group was also of lesser magnitude than in the saline-treated control rats with intact kidneys, the further slight rise of cholesterol following nephrectomy in these rats being ascribed to the effect of nephrectomy *per se* on the blood lipids (see Table I).

C. *The effect of albumin administration on plasma albumin and lipids of rats with previously established nephrosis*

Methods. Fourteen rats were each injected with one ml. of potent AKS and one week later all rats were bled for determination of total cholesterol. Each rat was subjected to cannulation of the lumbar vein as described above. During the ensuing 24-hour interval eight rats were infused with 7.0 ml. of 20 per cent solution of bovine albumin and the remaining six control animals were each injected with 7.0 ml. of 0.45 per cent saline solution. At the end of the 24-hour infusion interval all rats were bled for determination of total cholesterol and albumin concentrations.

Results. In Table IV it can be seen that the control nephrotic rats, following the infusion of saline solution, exhibited no significant change in their plasma cholesterol levels. However, as observed in the previous experiment, the infusion of albumin again considerably raised the plasma albumin levels above those of the controls. These rats, as might now be expected, exhibited a fall in their plasma cholesterol levels, in contrast to the control rats.

D. *The effect of stopping the infusion of albumin on the plasma lipids and cholesterol of rats injected with anti-kidney serum*

Methods. Ten rats were each injected with 1.0 ml. of potent AKS and bled for determination of total cholesterol 16 hours later. As described above, each rat then was subjected to cannulation of a lumbar vein and in the ensuing 24-hour interval, 5 of the rats were continuously infused with 7.0 ml. of the serum bovine solution (20 per cent) and 5 of the rats, serving as controls, were infused with 0.45 per cent saline solution. All animals then were bled for determination of total cholesterol. The infusion then was discontinued and 24 hours later both the experimental and control rats were bled for determination of total lipids, total cholesterol and albumin.

Results. The results of the analyses are shown in Table V in which it can be seen that 16 hours after injection of AKS only a moderate hypercholesteremia had developed in both the experimental and control rats. The average plasma cholesterol of the control rats receiving the saline infusion however, continued to rise progressively not only during the infusion interval but 24 hours after the latter's discontinuance, at which time marked hyperlipemia and hypoalbuminemia also were observed. In contrast, continuous infusion of the albumin solution into the experimental rats prevented further rise of the plasma cholesterol during the period of infusion. However, 24 hours after the infusion had been discontinued, their plasma cholesterol and lipids rose to levels comparable to those observed in the control rats. At this time too, the plasma albumin content of both groups of rats was approximately the same. By comparison with the data in Tables III and IV it can be seen that there was a rapid disappearance of the infused albumin (17, 18). This suggests of

course that, with the discontinuance of the albumin infusion, the experimental rats lost their artificially maintained elevated plasma albumin levels and in so doing, again became markedly hypercholesteremic and hyperlipemic.

DISCUSSION

Previously it was found that the hyperlipemia and hypercholesteremia of the nephrotic rat are of endogenous origin (2) and cannot be ascribed to any increased intestinal absorption (3) or decreased intestinal excretion (5). Moreover it was shown that the hepatic synthesis of cholesterol, the major source of the plasma cholesterol (19) is not increased in the nephrotic rat (4). Furthermore, it was found that the rise of lipids and cholesterol in the nephrotic rat is an *isolated* accumulation confined to the plasma, apparently consequent to a failure in the usual transfer and egress of cholesterol and other lipids from the plasma into the liver (6). In other words the isolated rise of plasma lipids appears due to a "trapping" phenomenon in the plasma (8), an effect which is probably augmented by the rise of cholate which also occurs in the nephrotic state (1). There is an associated endogenous chylomicronemia (7).

The presently reported experiments indicate that this "trapping" phenomenon is intimately related to and indeed initiated by a primary renal derangement. This is understandable because apparently the total experimental nephrotic syndrome stems from some initial reaction between renal tissue and the anti-kidney serum. The failure of nephrotic hypercholesteremia and hyperlipemia to appear in the absence of renal tissue was demonstrated previously by Heymann and Hackel (20). The present observations confirm their finding that viable renal tissue must be present for the initial renal reaction leading to the development of nephrotic hypercholesteremia and hyperlipemia in the experimental animal. The data further indicate, however, that the rise of plasma lipids will not occur, despite the demonstrated occurrence of the AKS-induced renal damage, unless an *external* loss of some plasma constituent occurs in the urine, either as a result of or in association with this renal damage. This was shown by the finding that if the *external* loss of urine by the kidney was prevented by ureteral ligation or ureter-vena caval anastomosis, the development of nephrotic hypercholesteremia and hyperlipemia was prevented despite the occurrence of the typical lesions both in the glomerulus and tubules.

These observations therefore indicate that the rise of plasma lipids is initiated by the external renal loss of some plasma constituent via the urine. The present observations further suggest that this particular substance lost in the urine is plasma albumin, because when *sufficient* albumin was given to AKS-injected rats to overcome the plasma deficit otherwise resulting from its rapid renal loss (17), hyperlipemia and hypercholesteremia either were prevented or markedly inhibited during the induction stage as well as ameliorated in the previously established nephrotic state. Furthermore, the subsequent rapid disappearance of the infused albumin from the circulation (17, 18) of some of these rats was associated with a concomitant rapid rise of serum lipids and cholesterol of similar magnitude to that of the control nephrotic rats.

The induced rise of plasma albumin to normal or greater levels was not associated with a fall of the plasma lipids and cholesterol entirely to normal concentrations in all rats. This may be due to the fact that bovine serum albumin, because of its ready availability, was used in these studies instead of concentrated rat serum albumin, and the physico-chemical state of such albumin is somewhat altered during the procedures used in its preparation and purification. Moreover, the egress of *excess* lipid from the circulation is a gradual process even in the normal animal (21), and a similar situation would obtain in the nephrotic rat despite alleviation of the metabolic block which otherwise prevents the usual egress of cholesterol and other lipids from the plasma in such rats.

Various observers (22–31) have failed to find a close inverse correlation between lowered plasma protein and elevated lipid content in both clinical and experimental nephrosis. However, determinations of total protein, rather than of the albumin fraction, generally were done and since globulins rise in nephrotic plasma (32), conclusions based upon such determinations may not be valid. Indeed, when the correlation between ele-

vated plasma lipids and lowered *albumin* rather than total protein has been studied, a much closer inverse relationship has been found in nephrotic patients (33, 34). A rise of plasma albumin, when induced by ACTH in nephrotic patients, is associated with a fall of plasma cholesterol regardless of the pattern of diuresis (35), and during clinical remissions in nephrotic patients it has been observed that shifts of plasma cholesterol and albumin occur together, both reaching normal levels nearly simultaneously (34). Moreover, no significant hyperlipemia and hypercholesteremia are observed as a consequence of the renal damage occurring in glomerular nephritis (25, 26) or in the many diverse states in which the nephrotic state may occur (35) unless there is excessive urine protein loss (26, 36, 37). In other words, the common denominator for "nephrotic" lipemia does not appear to be the renal lesion *per se* but the *external* renal loss of plasma albumin sufficient to induce hypoalbuminemia. This is consonant with the finding that a similar inverse plasma lipid-protein relationship has been observed clinically and experimentally in such other non-nephritic conditions in which plasma albumin is lowered as repeated blood loss in animals (38–40) and man (41), in patients with severe proteinuria due to constrictive pericarditis and renal thrombosis (37), in rats treated with DCA (42) or concomitantly with renin and adrenocortical steroids (42), in dogs fed low protein diets (43), and in animals made proteinuric by a nephrotoxin such as uranium (44).

Although the present data suggest that hypoalbuminemia *initiates* and maintains the processes both of hyperlipemia and hypercholesteremia, the sequential mechanism remains unclear. However, albumin is an essential component of lipemia-clearing processes (45) and the hydrolysis of plasma fats is markedly inhibited by the absence of adequate plasma albumin with available binding sites for fatty acids (45). The accumulation of plasma lipids may result from an inadequate supply of albumin for transport of these lipid breakdown products (46) from the plasma into the tissues and may in turn be responsible for the hypercholesteremia. In support of this last hypothesis is the recent finding in this laboratory (47) that an experimentally induced hyperlipemia quickly produces a hypercholesteremia. It thus is quite possible that the hypercholesteremia observed in the nephrotic state does not represent an initial or intrinsic error in cholesterol metabolism but a passively induced phenomenon secondary to the "heaping up" of lipids in the plasma consequent to the deficiency of plasma albumin. It is of considerable interest that Albrink, Man, and Peters (48) recently proposed the possibility of such a mechanism following their discovery of the preferential solubility of cholesterol in the neutral fat of plasma. It is also of interest that some years ago Addis (49), in considering the mechanism of nephrotic lipemia, hypothesized that, "In some such manner we can . . . see the milky plasma as the result of an absence of plasma protein molecules that have . . . transport function. Then the fatty acid molecules . . . would be left free in the water of the plasma and they would coalesce into the little fat globules that make the creamy emulsion we see." . . . "The lipemia may be a result of the decrease or absence of certain protein elements between whose layers fatty acid molecules usually lie recessed. We may suppose these proteins carry the fat in molecular dispersion and obviate their coalescence into droplets. Lipemia may thus be a manifestation of an inadequate transport system analogous to a traffic jam on the road. . . ."

SUMMARY

The prevention of *external* loss of urine in rats, either by ureteral ligation or by ureteral-vena caval anastomosis invariably prevented the hypercholesteremia and hyperlipemia otherwise observed following injection of rabbit anti-rat kidney serum. This suggests that the cholesterol and lipid abnormality observed in experimentally induced nephrosis is secondary to the *external* loss of one or more plasma substance.

Prevention or correction of the plasma albumin deficiency in the nephrotic rat was observed to inhibit markedly the hypercholesteremia and hyperlipemia otherwise occurring in association with the decreased plasma albumin induced by injection of anti-kidney serum.

It is suggested that the hyperlipemia and hypercholesteremia occurring in experimentally induced nephrosis result from the deficiency of circulating plasma albumin occurring in this disorder.

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THE ACTION OF HEPARIN IN THE PREVENTION OF PROTHROMBIN CONVERSION

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During physiological blood coagulation thrombin is formed from a precursor substance—prothrombin. According to the classical theory of blood coagulation as postulated by Morawitz in 1904, this conversion of prothrombin to thrombin is brought about by thromboplastin, acting in the presence of calcium (1). The thrombin then interacts with fibrinogen to produce fibrin. This theory may be expressed:

$$\text{Prothrombin} \xrightarrow[\text{Calcium}]{\boxed{\text{Thromboplastin}}} \text{Thrombin}$$

$$\text{Fibrinogen} \xrightarrow[\text{Thrombin}]{} \text{Fibrin}$$

Thromboplastin is defined in this study as a substance which is directly responsible for the conversion of prothrombin to thrombin in the presence of calcium (2). The thromboplastin was thought originally to be provided by damaged tissue, but it is now realized that when blood is withdrawn by clean venipuncture without tissue contamination it coagulates under the action of its own thromboplastin. This blood thromboplastin is now known to be of great potency (3), and is certainly essential for physiological hemostasis. The relative significance of these two thromboplastin systems in hemostasis has not been defined.

Many factors have recently been postulated as participants in blood thromboplastin formation. It is accepted for the present that four and possibly five components are required to react to form blood thromboplastin. These are antihemophilic globulin, platelets, Christmas factor (P.T.C.), factor V (labile factor, proaccelerin, plasma accelerator globulin) and possibly factor VII (prothrombin conversion accelerator, S.P.C.A., proconvertin, co-thromboplastin) (2). Antihemophilic globulin is deficient in hemophilia, platelets in thrombocytopenia, the Christmas factor in Christmas disease and factor V as a rare congenital anomaly and in some cases of hepatic disease. Factor VII is the predominant deficiency in coumarin drug therapy and though it undoubtedly is required in the tissue thromboplastin system, the evidence for its participation in blood thromboplastin is incomplete. These components can be substituted for the thromboplastin of the classical theory and the theory of blood thromboplastin expressed as follows:

$$\text{Prothrombin} \xrightarrow[\text{Antihemophilic globulin, Platelets, Christmas factor}]{\text{Factor V, Factor VII (tentative)}} \text{Thrombin}$$

$$\text{Fibrinogen} \xrightarrow[\text{Thrombin}]{} \text{Fibrin}$$

The order in which these components interact is uncertain but there is some evidence that antihemophilic globulin, platelets and the Christmas factor may interact to produce an intermediate product which is the equivalent of tissue extract, the other components subsequently entering the reactions involved in prothrombin conversion to thrombin (4). This concept of blood thromboplastin is in certain aspects analogous to the tissue mechanism, for in hemophilia, Christmas disease and thrombocytopenia the one-stage clotting time on the addition of tissue extract is normal, whereas deficiencies of factor V or VII cause a prolongation. This understanding of the components of blood thromboplastin may be incomplete as other factors have been suggested as being required, for example, P.T.A. (5), fourth thromboplastin component (6) and the Hageman factor (7).

With regard to the action of heparin in preventing coagulation, there is good experimental evidence that, while acting together with a cofactor in the albumin fraction of the plasma, it interferes with the thrombin-fibrinogen reaction (8, 9). It has also been suggested that heparin

interferes with the interactions resulting in thrombin formation (2, 10–12). Investigations on the action of heparin in preventing thrombin formation are difficult on account of the interference by the heparin with the thrombin-fibrinogen reaction. Thus, the components of blood thromboplastin can be incubated together with and without heparin and apparent interference with the production of thromboplastin demonstrated (3), but it can be argued that the results are merely a reflection of the heparin interfering with the thrombin-fibrinogen reaction.

The present investigation is concerned with the earlier stages of blood coagulation when subjected to the influence of heparin, using techniques where the heparin does not interfere with the final indicator system. It has been demonstrated that during blood coagulation under the influence of its own thromboplastin factor V, antihemophilic globulin and prothrombin are used up in the reaction (13–16). It is assumed that this consumption of factor V and antihemophilic globulin is due to their utilization in blood thromboplastin formation. Prothrombin consumption is the outcome of blood thromboplastin formation and the resulting conversion to thrombin.

METHODS

Preparation of reagents:

Prothrombin Fibrinogen Aluminum hydroxide (alumina) 3.8 per cent sodium citrate M/40 calcium chloride	As described by Biggs and Macfarlane (17)
Human brain extract	— As described by Brown and Douglas (18)

Centrifugation: All centrifugation was carried out at 4°C.

Glassware: All clotting times, whether of fibrinogen or plasma, were carried out in tubes of ½ inch diameter. The glassware was cleaned by standing in dichromate followed by rinsing in tap water.

Thrombin-fibrinogen dilution curve: As described by Biggs (19).

Measurement of prothrombin: As described by Douglas and Biggs (16). This technique is dependent on the separation of prothrombin from antithrombin and its activation thereafter by brain thromboplastin and calcium. The prothrombin was separated from the antithrombin by dilution and acidification to pH 5. The precipitate containing prothrombin was found to be freed not only from antithrombin, but also from heparin activity. There was, in consequence, no evidence of interference by heparin with the thrombin-fibrinogen reaction.

Measurement of factor V: As described by Douglas and Biggs (16), being a modification of the method of McClaughry and Seegers (20). Prothrombin is activated in an antithrombin free system by brain thromboplastin, calcium and factor V. The factor V is prepared from a normal and the test specimens. The test specimen is assayed by comparing the curve of activation with that of dilutions of the normal. The only difficulty in applying this technique to the present problem was the removal of the heparin from the appropriate test specimens and the prevention of its interference with the thrombin-fibrinogen reaction. It was found that treatment with alumina, which was employed in the preparation of the factor V from the specimens, removed the heparin by adsorption. This adsorption of heparin on alumina confirms the observations made independently by MacMillan and Brown (21).

Measurement of antihemophilic globulin: As described by Douglas and Biggs (16). This technique employs the thromboplastin generation test (22); it is dependent on the correction of thromboplastin generation of adsorbed hemophilic plasma by dilutions of adsorbed normal plasma. The curve of thromboplastin generation by mixtures of equal parts of adsorbed hemophilic and test specimens was compared with the dilutions of adsorbed normal in hemophilic plasma. A supply of hemophilic plasma was kept frozen at −20°C in a deep freeze. The heparin was removed by the process of adsorption on alumina.

Collection of specimens: The investigation was carried out on patients starting heparin therapy. Needles and syringes of identical size were used throughout. By venipuncture, using a wide bore needle (S.W.G. No 18), 30 ml. of blood was collected into a large syringe, care being taken to avoid frothing. This syringe was detached, the needle being left *in situ* and 10,000 units of heparin contained in another syringe, injected intravenously. Five minutes after the injection of heparin an identical specimen was collected by separate venipuncture from the other arm. A mixture was made immediately of 4.5 ml. of blood with 0.5 ml. of 3.8 per cent sodium citrate and further 4.5 ml. volumes of blood delivered into four identical graduated centrifuge tubes. These tubes were placed in a water bath at 37°C and at intervals of 15 minutes after collection 0.5 ml. of 3.8 per cent sodium citrate added and the contents of the tube mixed with a wooden applicator stick. In this way the process of clotting was arrested at fifteen-minute intervals after withdrawal of the blood. The tubes were left in the water bath for an hour to allow for the neutralization of any thrombin formed. After this the specimens were tested to determine the amounts of prothrombin, factor V and antihemophilic globulin present. In this way the pattern of utilization of these factors before and after the administration of heparin could be studied. The procedure described above was carried out on nine patients starting heparin therapy. In three of the observa-

TABLE I

The results of assays of prothrombin, factor V and antihemophilic globulin *

		Before heparin				After heparin			
		Time after venipuncture							
	Plasma	15 mins.	30 mins.	45 mins.	60 mins.	15 mins.	30 mins.	45 mins.	60 mins.
Prothrombin	100	79	51	10	0	100	100	100	100
	100	73	24	0	0	100	100	100	100
	100	74	10	0	0	100	100	100	100
Mean	100	75	28	3	0	100	100	100	100
Factor V	100	85	60	10	0	100	100	100	100
	100	70	40	0	0	100	100	100	100
	100	70	55	5	0	100	100	100	100
Mean	100	75	53	5	0	100	100	100	100
Antihemophilic globulin	100	100	15	0	0	100	100	100	100
	100	50	5	0	0	100	100	100	100
	100	60	5	0	0	100	100	100	100
Mean	100	70	8	0	0	100	100	100	100

* The mean of the three observations on each of these factors is also shown. The assays were made on plasma collected before giving heparin and on specimens citrated at 15, 30, 45, and 60 minutes following withdrawal of blood by venipuncture, both before and after the intravenous administration of heparin. The results are expressed as percentages of the amount of each of the factors present in the plasma before giving the heparin.

tions, prothrombin was studied, in three factor V and in the last three antihemophilic globulin.

RESULTS

The details of each of the assays is shown in Table I together with the mean of these results. It will be seen that after the administration of the heparin there was no utilization of any of these components up to one hour, whereas in the specimens collected before giving the heparin, all had been used up by the end of an hour.

DISCUSSION

Evidence has been produced to suggest that factor V and antihemophilic globulin are essential components of blood thromboplastin (2). During the clotting of blood under the influence of its own thromboplastin these factors disappear, presumably in consequence of their utilization in the formation of blood thromboplastin (16). In the presence of heparin, factor V and antihemophilic globulin are not used up. This can be interpreted as a consequence of interference with thromboplastin formation.

The investigation has shown that in the presence of heparin there is defective consumption of prothrombin. This is a further, non-specific, indication of failure in the blood thromboplastin system.

The techniques used in the investigation have employed methods for the removal of heparin from the incubation mixtures. There was in consequence no interference by heparin with the indicator system, the thrombin-fibrinogen reaction. The efficacy of the methods for the removal of heparin was confirmed by the demonstration that all the specimens collected after the administration of the heparin contained as much prothrombin, factor V and antihemophilic globulin as the plasma before administration of heparin. Any traces of heparin left in the incubation mixtures would have resulted in lower readings being obtained.

SUMMARY

1. Therapeutic doses of heparin have been shown to result in failure of utilization of antihemophilic globulin and factor V, believed to be essential components for blood thromboplastin formation. The consequent inability to convert prothrombin to thrombin has been demonstrated. The experimental results described are not the consequence of heparin acting on the indicator system.

2. This evidence of complete interference with prothrombin conversion and utilization of blood thromboplastin components may possibly indicate that this represents the main action of heparin rather than interference with the thrombin-fibrinogen reaction.

ACKNOWLEDGMENTS

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BILIRUBIN–PROTEIN LINKAGES IN SERUM AND THEIR RELATIONSHIP TO THE VAN DEN BERGH REACTION [1]

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Innumerable theories have been advanced to explain the direct and indirect van den Bergh reactions of serum bilirubin. However, only three are still current. These postulate that the two types of diazotization are dependent on (a) differences in the chemical structure of bilirubin, (b) the presence of a catalyst that facilitates diazotization in the absence of alcohol, or (c) differences in the linkage of bilirubin to protein.

The theory that the variable behavior of bilirubin is in some way related to the nature of its linkage with protein has always had the greatest number of proponents, and has had an important influence in the formulation of current concepts of regurgitation and retention jaundice (1, 2). As originally conceived (3) indirect-reacting bilirubin was assumed to be a protein complex which was split as it passed through the liver cells, so that the direct-reacting pigment in bile and that found in the serum under certain conditions was thought to be present in an unbound state. However, it is now known from studies based on dialysis (4), ultra-filtration (5, 6), ultracentrifugation (7), and electrophoresis (6–8), that both types of pigment are linked to protein. This has led to several revisions of the theory. Accordingly, it has been suggested that, while both types of bilirubin are bound to albumin, the complex is dissociable in the case of direct-reacting serum, and stable, possibly because of a valence bond, in the case of the indirect-reacting type (6). As an alternative, it has been proposed that the two types of pigment are linked to different proteins, the direct-reacting type being bound to albumin and the indirect to globin (9, 10) or to an alpha-globulin (11). Watson (1) has incorporated both ideas into what is, perhaps, the most widely accepted concept at the present time. According to this view indirect-reacting bilirubin is a stable globulin complex derived from hemoglobin. On passing through the liver cells bilirubin is split off and excreted into the bile as a sodium salt which yields the direct van den Bergh reaction. Under conditions leading to the regurgitation of bile into the blood, sodium bilirubinate is loosely bound by albumin, but continues to react directly. This theory is based on the assumption that: *1*) in the breakdown of hemoglobin to bilirubin, globin remains firmly attached to the ruptured porphyrin nucleus until it traverses the liver cells, *2*) the electrophoretic mobility of bilirubin-globin is identical with that of albumin, the fraction of serum in which most, if not all, the bilirubin is usually found (*7*, 8), *3*) sodium bilirubinate reacts directly both in the presence and absence of albumin, and *4*) the addition of alcohol in the indirect reaction facilitates diazotization by releasing bilirubin from its protein complex. Little direct evidence has been presented in support of this hypothesis, and, indeed, a number of observations appear to be inconsistent with it.

Early electrophoretic studies indicated that all the bilirubin in serum was bound to albumin (6). However, on fractionating the plasma proteins under special conditions Cohn (12) found that the alpha$_1$-globulin concentrated in fraction V-1 contained a pigment which gave the indirect van den Bergh reaction. Later, Gray and Kekwick (8) confirmed the presence of bilirubin in alpha-globulin electrophoretically. However, they emphasized that the bulk of the pigment was always associated with albumin, and that the fraction bound to globulin was constant in amount and independent of the type of van den Bergh reaction. On the basis of ammonium sulfate fractionation

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studies, Najjar and Childs (11, 13) also reached the conclusion that a fraction of serum bilirubin was bound to globulin. However, in contrast to Cohn (12) and Gray and Kekwick (8), they suggested that all of the indirect-reacting pigment in serum was associated with a globulin, while all of the direct-reacting type was bound to albumin.

The present investigation was undertaken to re-evaluate the conflicting evidence pertaining to the protein-linkage of bilirubin in serum, and its relationship to the van den Bergh reaction. Filter-paper electrophoresis, which was the principal method employed, provided a convenient means of studying many of the problems involved. In particular it made it possible to characterize the properties of the pigments isolated from each of the protein fractions, and to compare them with those present in the original serum. Due consideration was given to the fact that all sera contain both direct- and indirect-reacting bilirubin, and that the qualitative van den Bergh reaction, used to identify the pigments in many similar investigations, merely distinguishes between low and high concentrations of direct-reacting pigment (1, 14).

METHODS

Serum samples. Blood was drawn from patients with various types of jaundice and allowed to coagulate at room temperature. Following centrifugation the supernatant serum was analyzed for its content of direct-reacting ("one-minute") and total bilirubin by a modification (14) of the Malloy-Evelyn method (15). The remaining serum was then stored at 5 to 10° C for 8 to 10 hours before being subjected to electrophoresis. In a few experiments serum was stored in the frozen state at −20° C for periods up to several weeks. Preliminary investigation revealed that the electrophoretic mobilities of bilirubin and protein did not change during such storage. The total bilirubin concentration fell slightly, but there was no alteration in the ratio of the direct- and indirect-reacting fractions.

Electrophoresis. Electrophoretic analyses were carried out at room temperature by the filter-paper method described by Grassmann and Hannig (16). This entailed the use of (a) Whatman No. 1 filter-paper strips (4 × 40 cm.) stretched horizontally between buffer-filled electrode wells in a shallow, glass- and plastic-enclosed air-chamber,[3] (b) Michaelis' buffer (sodium veronal, sodium acetate and HCl) with a pH of 8.6 and ionic strength of 0.1, and (c) a voltage of 110 for 14 to 16 hours yielding a current of approximately 1.0 mA through each of the strips. Three chambers, each containing a pair of strips, were usually run simultaneously.

[3] Obtained from Bender and Hobein, Munich, Germany.

One strip in each chamber was streaked with 0.10 ml. of serum and was used for the separation and identification of the pigment in each of the protein fractions. This was found to be the smallest volume which permitted accurate qualitative and quantitative analysis of the pigments. Unfortunately, resolution of the protein bands was not optimal, so that it was found desirable to run a control strip in each chamber streaked with 0.01 ml. of serum. This yielded sharply defined protein bands which facilitated identification of the peaks in the denser strip. It should be noted, however, that the mobilities of protein and pigment were always compared on the same strip, since their absolute mobilities on the two strips were not always identical. Better definition of the protein peaks could be achieved with large volumes of serum by using heavier filter-paper. However, eluates from such paper were invariably clouded by a fine pulp which could not be removed by filtration or centrifugation.

The electrophoretically separated protein fractions were stained and then quantitated either by direct densitometry or by spectrophotometry following elution. The pigments were studied in a similar manner, except that preliminary staining was omitted. Since it was essential to analyze the same strip for both protein and bilirubin it was not possible to use both the densitometric and elution methods in each instance. Therefore, each strip was subjected to one of two combined procedures: 1) direct densitometry of the pigment bands followed by staining and subsequent densitometry of the protein fractions, or 2) division of the strip into two longitudinal sections, measuring 8 and 32 mm. in width, respectively, followed by staining and direct densitometry of the narrower strip for the localization of the protein bands, and elution of the unstained broader strip for bilirubin analysis. The former method permitted more accurate determination of the relative mobilities of the pigment and protein fractions, while the latter made it possible to establish the chemical nature of the pigments. Accordingly most experiments were carried out in duplicate using both combined procedures.

Staining of protein. Following air-drying and heat-fixation, the strips were stained with Amidoschwarz 10B and washed in acetic acid-methanol as recommended by Grassmann and Hannig (16).

Densitometry. The location and density of the pigment bands and the stained protein fractions were determined by scanning the strip at 1 mm. intervals through a 25 × 1 mm. slit in a Photovolt densitometer (Model 525). The recorded density readings were then plotted against the migration in millimeters and the points joined to form smooth curves. When the strip was split longitudinally, as described above, the narrow, stained protein strip was scanned at 1 mm. intervals through a 6 × 1 mm. slit.

The protein-stained strips were cleared in alpha-bromnaphthalene and paraffin oil (16) before scanning, a procedure which decreased the density and permitted more accurate densitometry. This was omitted in the case of the unstained strips to be scanned for pigment.

It was found that the relationship between the areas of the peaks delineated by direct densitometry and the con-

centration of protein in the individual fractions was not strictly linear, so that the patterns obtained did not provide an accurate estimate of relative concentrations. Nevertheless, this in no way detracted from the precision of the method in determining the relative mobilities of the protein and pigment fractions.

Determination of eluted bilirubin. Attempts to identify and quantitate bilirubin on filter paper by diazotization and direct densitometry were unsuccessful, since the color evolved was never typical of azobilirubin, and was usually of too low intensity to measure accurately. It was necessary, therefore, to devise a technique which permitted both quantitative analysis and study of the absorption spectrum of the small amounts of pigment present in the eluates of 5 mm. filter-paper segments. This was accomplished by modifying the Malloy-Evelyn method (15) taking advantage of the greatly increased sensitivity afforded by the use of long pathway micro-cuvettes (50 × 4 mm.) in a Beckman DU spectrophotometer (17).

Each 5 mm. segment of filter-paper was immersed in 2 ml. of distilled water for one hour, following which the eluate was divided into two 1-ml. aliquots. To one was added 0.2 ml. of diazo blank and to the other 0.2 ml. of freshly prepared diazo reagent. The mixtures were agitated, transferred to micro-cuvettes and then read in the spectrophotometer at a wave length of 540 mμ in precisely one minute. For the total bilirubin determination the contents of each cuvette were removed by pipette and returned to their original containers. To each of these was then added 1.2 ml. of absolute methanol with thorough mixing. At the end of one-half hour the cuvettes were refilled and read in the spectrophotometer as before.

To characterize further the nature of the pigments in each of filter-paper segments, the absorption spectrum of each eluate was studied both before and after diazotization.

RESULTS

Direct densitometry of pigments in filter-paper electrophoretic patterns of icteric serum

All strips showed a sharply defined yellow band which coincided precisely with the position of the albumin peak, irrespective of whether the bilirubin content of the original serum was predominantly of the direct- or indirect-reacting type. On the basis of its absorption spectrum and subsequent behavior on diazotization, the pigment eluted from this band was identified as bilirubin. A second inconstant pigment of a pale reddish-brown hue was occasionally observed in the region of the alpha and beta globulins. This gave a positive guaiac reaction, had the absorption spectrum of hemoglobin, and therefore, was interpreted as an artifact due to hemolysis. A typical pattern showing both bands and their relationship to the separated protein fractions of serum is illustrated in Figure 1A.

The pigment associated with the albumin fraction, and presumed to be bilirubin, revealed a well

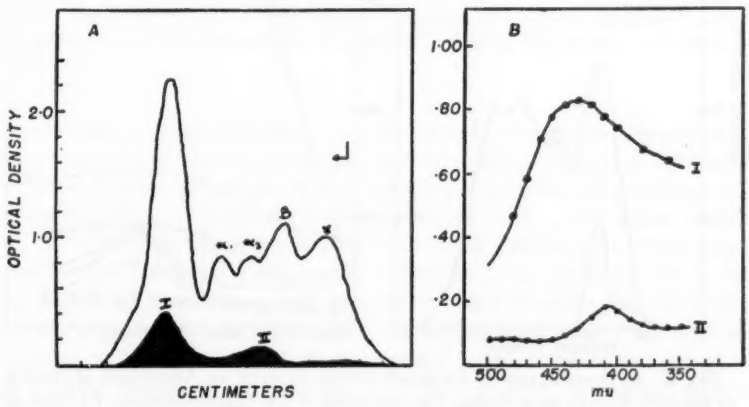

FIG. 1. A. ELECTROPHORETIC PATTERN OF SERUM IN OBSTRUCTIVE JAUNDICE SHOWING TWO PIGMENT PEAKS AND THEIR RELATIONSHIP TO THE PROTEIN FRACTIONS

Both curves obtained by direct densitometry. Serum bilirubin: direct 25.0 mg., total 48.6 mg. per 100 ml.

B. ABSORPTION CURVES OF ELUTED PIGMENTS ILLUSTRATED IN FIGURE 1A, SHOWING ABSORPTION MAXIMUM AT 430 mμ IN PIGMENT I (BILIRUBIN) AND AT 410 mμ IN PIGMENT II (HEMOGLOBIN)

defined absorption maximum at 430 instead of 460 mμ (Figure 1B), the value expected for serum bilirubin (18). Errors in instrumentation did not appear to account for this discrepancy, since the accuracy of the wave-length scale of the spectrophotometer employed was verified with the aid of a mercury vapor lamp. Possibly the presence of interfering substances, such as biliverdin, was responsible. However, the bulk of the pigment eluted behaved as bilirubin on diazotization.

The pigment associated with the alpha and beta globulins, and shown to be hemoglobin, had an absorption maximum at 410 mμ (Figure 1B), and could, therefore, be readily distinguished from bilirubin.

Spectrophotometric analysis of eluted pigments

An attempt was made to demonstrate the presence of bilirubin in the globulin fractions of serum by analyzing the absorption spectrum of each of the eluates of serial transections of the entire electrophoretic pattern. The results of two typical experiments on serum with predominantly direct- and indirect-reacting bilirubin, respectively, are illustrated in Figures 2 and 3. It will be noted that in both instances the only pigment with an absorption maximum at 430 mμ was found in the region of the albumin peak. It was not possible to exclude the presence of traces of bilirubin in the globulins, since the analytical procedure employed was insensitive to concentrations below 0.9 micrograms per ml. of eluate, an amount equivalent to 1.80 mg. per 100 ml. of original serum. However, it was evident that the bulk of the pigment could be accounted for in the albumin fraction in all cases.

Diazotization of eluted pigments

To increase the sensitivity and specificity of the method of detecting bilirubin in serial sections of filter-paper electrophoretic patterns, the eluates obtained were subjected to diazotization and ana-

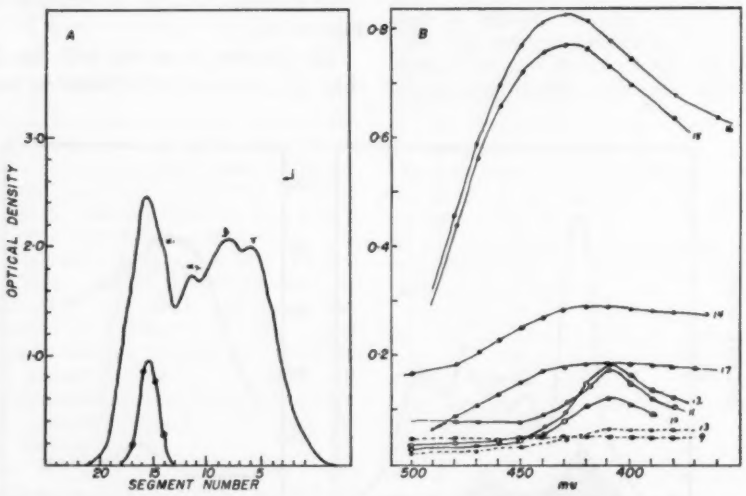

FIG. 2. A. DISTRIBUTION OF BILIRUBIN (PIGMENT WITH AN ABSORPTION MAXIMUM AT 430 MΜ) ELUTED FROM SERIAL TRANSECTIONS OF AN ELECTROPHORETIC PATTERN OF SERUM IN OBSTRUCTIVE JAUNDICE

Serum bilirubin: direct 8.0 mg., total 17.1 mg. per 100 ml.

B. ABSORPTION CURVES OF INDIVIDUAL ELUATES OF 5 MM. SEGMENTS OF THE PATTERN ILLUSTRATED IN FIGURE 3A

Note the maximum at 430 mμ (bilirubin) in segments 14–17 corresponding to the albumin peak and at 410 mμ (hemoglobin) in segments 10–12 corresponding to alpha$_2$ and beta-globulins. The remaining segments, of which numbers 9 and 13 are representative, showed no maximum.

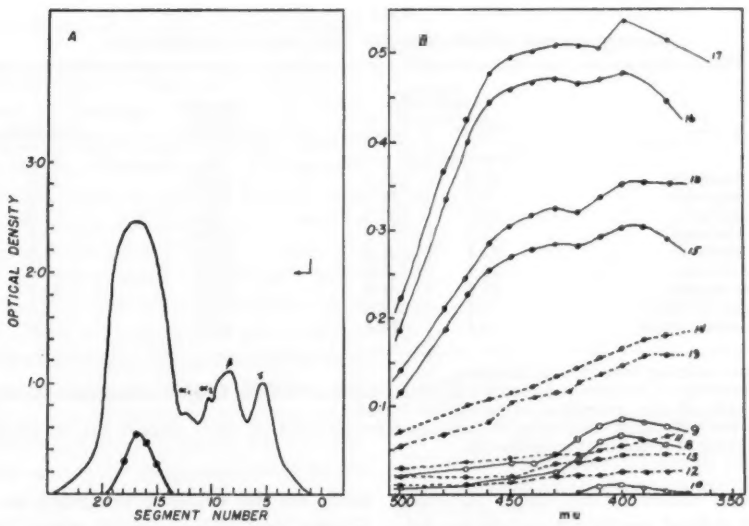

FIG. 3. A. DISTRIBUTION OF BILIRUBIN (PIGMENT WITH AN ABSORPTION MAXIMUM AT 430 Mμ) ELUTED FROM SERIAL TRANSECTIONS OF AN ELECTROPHORETIC PATTERN OF SERUM IN ERYTHROBLASTOSIS FETALIS

Serum bilirubin: direct 0.4 mg., total 18.6 mg. per 100 ml.

B. ABSORPTION CURVES OF INDIVIDUAL ELUATES OF 5 MM. SEGMENTS OF THE PATTERN ILLUSTRATED IN FIGURE 4A

Note the maximum at 430 mμ (bilirubin) in segments 15–18 corresponding to the albumin peak, and at 410 mμ (hemoglobin) in segments 8–10 corresponding to beta-globulin. The remaining eluates, of which only five are illustrated, show non-specific absorption curves. It will be noted that there is a second maximum at 400 mμ in the eluate from segments 15–18, probably indicating the presence of methem-albumin.

lyzed spectrophotometrically for azobilirubin. The technique used was capable of demonstrating as little as 0.1 microgram of bilirubin per ml. of eluate, a concentration equivalent to 0.24 mg. of direct or 0.48 mg. of indirect-reacting pigment per 100 ml. of original serum. However, it was found that, while this degree of sensitivity could be achieved in recovery experiments with eluates of serum freshly applied to buffer-moistened filter-paper, there was a highly significant loss (average 65 per cent) of diazotizable bilirubin following electrophoresis (Table I), thereby reducing the effective sensitivity of the method to approximately 0.3 microgram per ml. of eluate, a concentration equivalent to 0.72 mg. of direct- or 1.44 mg. of indirect-reacting bilirubin per 100 ml. of original serum. This apparent loss of bilirubin was probably due to oxidation, since a similar decrease in diazotizable pigment was observed in a control serum applied to moistened filter-paper and exposed to the same atmosphere for a similar length of time. Adsorption of bilirubin in the trail of the proteins during their migration did not appear to be a significant factor, since lengthening the trail by prolonging the electrophoresis did not increase the loss of bilirubin over that of a control serum on moistened paper.

Nine samples of serum were investigated. These were obtained from patients with jaundice, and contained varying proportions of direct- and indirect-reacting bilirubin (Table I). As illustrated by the two representative patterns in Figure 4, the peaks of both direct- and indirect-reacting bilirubin invariably coincided with that of albumin. In carrying out these experiments non-specific absorption was excluded by analyzing the absorption

TABLE I

Recovery of serum bilirubin from filter paper following electrophoresis

Type of serum	Bilirubin concentration		Bilirubin applied to strip†	Bilirubin recovered	
	Direct*	Total			
	mg. per cent	*mg. per cent*	*mcg.*	*mcg.*	*per cent*
Infectious hepatitis	11.5	26.5	21.2	19.9	90‡
Infectious hepatitis	6.3	15.8	12.6	4.7	37
Laennec's cirrhosis	11.1	21.8	17.4	7.0	40
Laennec's cirrhosis	12.7	28.2	22.6	5.7	25
Laennec's cirrhosis	12.7	28.2	22.6	4.8	21
Laennec's cirrhosis	11.5	29.6	23.7	7.8	33
Obstructive jaundice	13.4	28.9	23.1	12.7	55
Erythroblastosis fetalis	0.4	18.6	14.9	4.7	32
Erythroblastosis fetalis	0.3	18.4	14.7	4.9	33

* Prompt direct-reacting (one-minute) fraction.

† Amount remaining on the filter-paper strip following removal of an 8 mm. longitudinal segment for protein analysis; original width of strip 40 mm., volume of serum applied 0.1 ml.

‡ This value was omitted in calculating the average recovery (34.5 per cent), since it was not possible to achieve this degree of recovery in subsequent experiments.

spectrum of each eluate. Only those pigments showing a well defined maximum at 540 mμ following diazotization were considered bilirubin.

Experiments with globin

The concept that the type of van den Bergh reaction in serum depends on whether bilirubin is bound to albumin or globin is based almost entirely on the evidence presented by Fiessinger, Gaydos, and Polonovski (9, 10). These investigators have reported that neutralized solutions of bilirubin react directly in the presence of serum albumin and indirectly in the presence of globin, and that it is possible to convert the direct-reacting type to the indirect by incubating serum at ice-box temperature in the presence of globin, a change

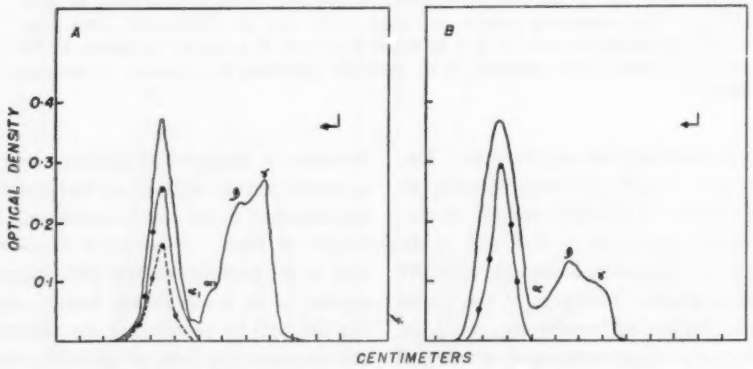

FIG. 4. A. DISTRIBUTION OF DIRECT-REACTING (·---·) AND TOTAL (·——·) BILIRUBIN IN ELUATES FROM SERIAL TRANSECTIONS OF AN ELECTROPHORETIC PATTERN OF SERUM IN LAENNEC'S CIRRHOSIS

The indirect-reacting fraction of bilirubin is represented by the area between the two pigment curves beneath the albumin peak. Serum bilirubin: direct 12.7, total 28.2 mg. per 100 ml.

B. DISTRIBUTION OF INDIRECT-REACTING BILIRUBIN (·——·) IN ELUATES FROM SERIAL TRANSECTIONS OF AN ELECTROPHORETIC PATTERN OF SERUM IN ERYTHROBLASTOSIS FETALIS

No direct-reacting pigment could be demonstrated. Serum bilirubin: direct 0.4, total 18.6 mg. per 100 ml.

presumably due to a shift of pigment from albumin to globin.

In a preliminary experiment, based on the technique described by Fiessinger, Gaydos, and Polonovski (9) the qualitative van den Bergh reaction of bilirubin-protein mixtures was investigated. Bilirubin was dissolved in 0.01 N NaOH at a concentration of 40 mg. per 100 ml. and added in amounts ranging from 0.2 to 0.6 ml. to 2-ml. aliquots of 0.35 per cent albumin [4] or globin.[5] With the aid of a glass electrode the mixtures were then brought to a pH of 7.0 and allowed to stand for one hour, following which 0.5 ml. of freshly prepared diazo reagent was added to each tube. An indirect reaction was obtained in all tubes, indicating that the reactivity of bilirubin was not dependent on the nature of the protein with which it was associated.

To characterize the behavior of these bilirubin-protein mixtures more precisely, their diazotization reaction curves were then investigated spectrophotometrically. The solutions tested were prepared and analyzed as follows: (a) to 8-ml. aliquots of 0.35 per cent albumin and globin were added 2-ml. portions of a 10 mg. per cent solution of bilirubin, (b) the mixtures were then brought to a pH of 7.0 and allowed to stand for one hour; to maintain the same concentrations of bilirubin and protein in both mixtures their volumes were equalized with water following neutralization, (c) two 3-ml. aliquots of each mixture were then transferred to 1-cm. cuvettes to which were added 0.6-ml. portions of freshly prepared diazo reagent and blank, respectively, (d) optical density was then measured at frequent intervals for a period of 30 minutes in a Beckman DU spectrophotometer at a wave-length of 540 mμ, (e) at the end of this period an equal volume of absolute methanol was added to each cuvette and the optical density measured as before. The final concentrations of protein and bilirubin before diazotization were 223 and 1.82 mg. per 100 ml., respectively. A control solution of bilirubin without protein was analyzed simultaneously. It had a slightly lower concentration of bilirubin, 1.43 mg. per 100 ml., since it required a larger volume of acid for neutralization.

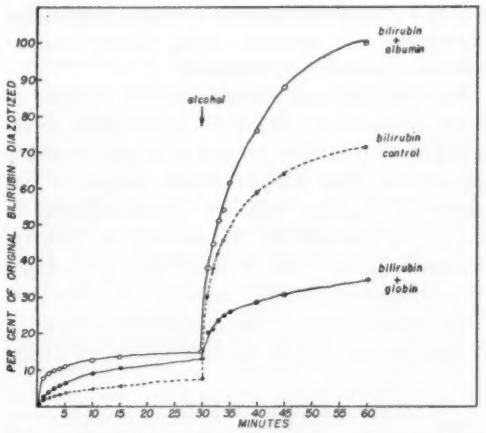

FIG. 5. DIAZOTIZATION REACTION CURVES OF NEUTRALIZED SOLUTIONS OF BILIRUBIN IN THE PRESENCE OF GLOBIN AND ALBUMIN

The diazotization curves obtained in this experiment are illustrated in Figure 5. It will be observed that in all three solutions less than 10 per cent of the bilirubin reacted directly during the first minute, and less than 15 per cent in 30 minutes, behavior characteristic of indirect-reacting serum (14). When alcohol was added, there was a prompt increase in the rate of diazotization in all tubes. However, at the end of 30 minutes there was a marked difference in the amount of azobilirubin recovered in each of the solutions. While all of the pigment reacted in the albumin mixture, only 70 per cent reacted in the control solution and less than 35 per cent in the globin mixture. The loss of reactivity in the control solution was undoubtedly due to the oxidation of bilirubin, an effect which was inhibited by combining the pigment with albumin (20). The fact that even less azobilirubin was recovered in the presence of globin than in the control solution indicated that globin was not only a less effective anti-oxidant than albumin, but that it actually increased the loss of diazotizable pigment. A clue to the mechanism involved was afforded by the observation that the bilirubin-globin solution became slightly turbid when it was neutralized. This suggested the possibility that the mixture had partially precipitated when the pH was brought

[4] Crystallized human serum albumin obtained from the Harvard University Laboratory of Physical Chemistry, through the courtesy of Dr. J. L. Oncley.

[5] Prepared in the authors' laboratory by the method of Anson and Mirsky (19).

to 7.0, a suspicion that was confirmed when the experiment was repeated using higher concentrations of protein and bilirubin.

The solubility and bilirubin-binding capacity of globin at the normal pH of blood were then determined, since it seemed possible from the preceding experiments that normal serum might be incapable of holding sufficient bilirubin-globin in solution to account for the amount of indirect-reacting bilirubin found in many hemolytic states. A modification of Martin's method (21) was used to obtain these data. This entailed the dialysis of a 0.365 per cent globin solution against an excess of bilirubin in phosphate buffer (pH 7.4, ionic strength 0.1) containing ascorbic acid, and the determination of its bilirubin and protein content following a period of equilibration at 10° C. A control experiment, in which the cellophane sac contained buffer instead of globin solution, was carried out simultaneously. It was assumed that equilibrium had been reached when the bilirubin concentrations were identical on both sides of the membrane in the control experiment. This occurred in approximately 48 hours, at which time the buffer in both compartments of the control chamber had a bilirubin concentration of 0.40 mg. per 100 ml., based on direct measurements of absorption at 430 mμ in a Beckman DU spectrophotometer. At the same time, the globin mixture, which was now turbid due to precipitation of protein, was found to have a bilirubin content, after centrifugation, of 1.29 mg. per 100 ml. Since the concentration in the surrounding buffer was similar to that of the control, 0.37 mg. per 100 ml., it was assumed that 0.92 mg. of bilirubin per 100 ml. had been bound by the globin remaining in solution. By the Kjeldahl method the latter was found to be present in a concentration of 0.205 gm. per 100 ml., which was taken to be the approximate solubility of globin at pH 7.4. If these estimates of globin solubility and bilirubin-binding capacity are valid a bilirubin-globin complex could hardly account for more than 1 mg. of indirect-reacting bilirubin per 100 ml. at the normal pH of serum.

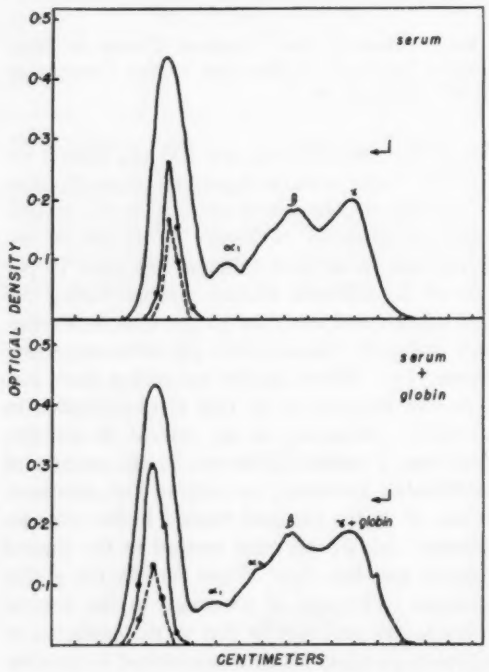

FIG. 6. EFFECT OF INCUBATING DIRECT-REACTING SERUM WITH GLOBIN AT 10° C FOR 18 HOURS

The upper panel shows the relationship of the eluted pigments to the electrophoretically separated proteins of 0.075 ml. of serum containing 4.5 mg. of direct-reacting and 10.8 mg. of total bilirubin per 100 ml. It will be noted that both the direct (·– – –·) and indirect-reacting (·——·) bilirubin are confined to the albumin fraction. The lower pattern, which was obtained with 0.150 ml. of an equal mixture of the same serum and 3.5 per cent globin following incubation, shows essentially the same ratio of direct- to indirect-reacting bilirubin and the absence of any shift of pigment to the globin peak.

On electrophoretic analysis the mobility of globin, which approximated that of a gamma globulin, differed from that of either direct- or indirect-reacting serum bilirubin. Moreover, when globin was added to an icteric serum containing a high proportion of direct-reacting bilirubin and was incubated at 10° C for 18 hours, there was no electrophoretic evidence of a shift of pigment to the globin peak, nor any increase in the relative concentration of indirect-reacting bilirubin (Figure 6), findings at variance with those reported by Fiessinger, Gaydos, and Polonovski (9).

The data presented strongly suggest that none of the bilirubin in serum is bound to globin. However, the evidence cannot be regarded as conclusive, since, as in all experiments involving the isolation and, particularly, the chemical manip-

ulation of globin, the question of denaturation arises. In both the present experiments and those described by Fiessinger, Gaydos, and Polonovski (9) globin was prepared and purified by the method of Anson and Mirsky (19). Although this material is thought to be undenatured, particularly since it can be recombined with heme to form hemoglobin (19) it is noteworthy that it has a molecular weight only one-fourth that estimated for the protein moiety of hemoglobin (22) and that the physical properties of the hemoglobin derived from it differ slightly from those of the natural product (23, 24). Moreover, in the present experiments the use of alkaline solutions of bilirubin may have denatured the globin further.

Experiments with alpha$_1$-globulin

Since the alpha$_1$-globulin of Fraction V-1 was found by Cohn (12) to give an indirect van den Bergh reaction, and was shown by Martin (21) to be capable of binding additional bilirubin, the possibility that the bilirubin in predominantly indirect-reacting serum was associated with this fraction was investigated. When Fraction V-1 [6] was added to a serum from a patient with hemolytic jaundice the expected increase in alpha$_1$-globulin was clearly discernible in the electrophoretic pattern; however, the peak obtained did not coincide with that of bilirubin. In addition, it was found that albumin had a greater affinity for bilirubin than the alpha$_1$-globulin of Fraction V-1. Thus, by means of electrophoresis it could be shown that both proteins were capable of combining with bilirubin *in vitro,* but that when an alkaline solution of bilirubin was added to a mixture of the proteins, the pigment migrated with the albumin exclusively, although undetected traces may have been present in alpha$_1$-globulin peak. It is reasonable to suppose, therefore, that there is a similar preferential binding of bilirubin by albumin *in vivo.* Moreover, on the basis of its known concentration of 0.05 per cent of plasma (12) and its bilirubin-binding capacity of 12 mg. per gm. (21), it was estimated that an alpha$_1$-globulin complex could not account for more than 0.05 mg. of indirect-reacting bilirubin per 100 ml. of serum.

[6] Obtained from the Harvard University Laboratory of Physical Chemistry through the courtesy of Dr. J. L. Oncley.

Ammonium sulfate fractionation of serum bilirubin

According to Najjar and Childs (11, 13) the ammonium sulfate fractionation patterns of serum bilirubin indicate that the direct-reacting type is bound to albumin, and the indirect to a globulin. However, they attach no importance to this difference insofar as the van den Bergh reaction is concerned, since they have found that the pigments retain their characteristic behavior on diazotization following their separation from protein.

Since Najjar's observations on the protein-linkages of bilirubin in serum were not in accord with those based on electrophoresis, an attempt was made to confirm the reported difference in the ammonium sulfate fractionation patterns of direct- and indirect-reacting bilirubin. The technique employed was virtually identical with that described by Najjar and Childs (13). Four-tenths of a ml. of serum was pipetted into each of 12 celluloid centrifuge tubes. Sufficient water, bromphenol blue and saturated ammonium sulfate were then added to yield ammonium sulfate concentrations ranging from 32 to 72 per cent saturation, and a bromphenol blue concentration of 0.04 mg. per ml. of serum in a total volume of 10 ml. The salt was added dropwise with stirring to ensure adequate mixing. The samples were then stored in the refrigerator at 5° C for one hour, following which they were centrifuged for 30 minutes at 40,000 RPM.

The sediment in each tube was redissolved in 5 ml. of saline and analyzed for bilirubin and bromphenol blue by measuring its absorption at 460 mμ and 605 mμ, respectively, in a Beckman DU spectrophotometer. Corresponding concentrations of ammonium sulfate and water served as blanks. Due to the development of turbidity, it was not possible to check the results by diazotization, as reported by Najjar and Childs (13). Bilirubin determinations were also carried out on the supernatants, which revealed that excellent recoveries of total pigment were being achieved.

Seven serum samples were studied, four of the direct-reacting type from patients with various forms of obstructive or hepatocellular jaundice, and three of the indirect type from infants with erythroblastosis fetalis. In all instances the serum was virtually hemoglobin-free. Nevertheless, in analyzing the mixtures for bilirubin a correction

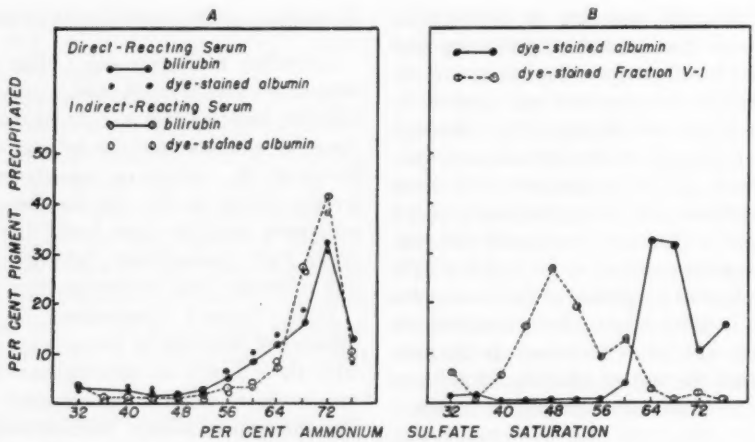

FIG. 7. A. AMMONIUM SULFATE FRACTIONATION PATTERNS OF BILIRUBIN AND ADDED BROMPHENOL BLUE IN DIRECT- AND INDIRECT-REACTING SERUM

The former contained 19.7 mg. of bilirubin per 100 ml., of which 10.6 mg. reacted directly, the latter 22.1 mg. per 100 ml., of which 0.6 mg. reacted directly.

B. AMMONIUM SULFATE FRACTIONATION PATTERNS OF BROMPHENOL BLUE IN 4 PER CENT SOLUTIONS OF SERUM ALBUMIN AND COHN'S FRACTION V-1

for hemoglobin was made, as suggested by Najjar (11) by using the following formula:

$$\text{True absorption at } 460\ m\mu = A_{460} - \frac{A_{420} - (A_{460} \times 0.86)}{4},$$

where A_{460} = absorption of bilirubin at 460 mμ, and A_{420} = absorption of bilirubin at 420 mμ.

In all but one instance a direct- and an indirect-reacting serum were analyzed simultaneously to maintain identical conditions. It was found, in contrast to the observations of Najjar and Childs (13), that the fractionation pattern of precipitated bilirubin invariably coincided with that of bromphenol blue-labelled albumin, both in direct- and indirect-reacting serum (Figure 7A). As a further check on the apparent similarity of the precipitation patterns of direct- and indirect-reacting bilirubin to that of albumin, ammonium sulfate fractionation was carried out on bromphenol blue-stained solutions of human serum albumin [7] and Cohn's fraction V-1. This appeared to be an essential control, since it was found that unbound dye could always be detected in the presumably protein-free supernatant at 76 per cent ammonium sulfate saturation, even when the lowest concentration of dye recommended by Najjar and Childs (13) was used, indicating that an excess was always available for staining of globulin. Fraction V-1 was selected for testing since Najjar (11) had suggested that indirect bilirubin might be associated with an alpha-globulin. As can be seen in Figure 7B, the fractionation pattern of dye-labelled albumin approximated that of protein-bound bilirubin in both direct- and indirect-reacting serum, and that the pattern of dye-labelled Fraction V-1 resembled neither.

No explanation can be offered for the discrepancy between the results reported by Najjar and Childs (13) and those obtained in this laboratory. However, the latter would appear to be more consistent with the other evidence presented indicating that bilirubin is bound to albumin in both types of serum.

Effect of pH change on the electrophoretic mobility of serum bilirubin

If different proteins bind the two types of bilirubin in serum, it must be assumed that their electrophoretic mobilities are identical, as in the case of the pigments. While it is possible for hetero-

[7] Salt-poor concentrated human serum albumin obtained from the American Red Cross through the courtesy of Dr. S. T. Gibson.

geneous proteins to migrate at the same rate under certain conditions, it is highly improbable that they will do so over a wide range of pH if they differ as markedly in amino acid composition as albumin and globin (25). For that reason the electrophoretic patterns of sera containing predominantly direct- and indirect-reacting bilirubin, respectively, were compared in buffers ranging in pH from 2.0 to 9.0. Both samples were run simultaneously in the same chamber to ensure identical conditions. The buffers, which had an ionic strength of 0.1, were made up of glycine-HCl for pH 2.0 and 3.0, sodium acetate-acetic acid for pH 4.0 and 5.0, Na_2HPO_4–NaH_2PO_4 for pH 6.0 and 7.0, and sodium veronal-HCl for pH 8.0 and 9.0. In a duplicate experiment the ionic strength of the buffer salt was kept at 0.02 and brought to 0.1 with sodium chloride, as recommended by Miller and Golder (26). This modification did not appear to alter the mobilities of either protein or bilirubin. The position of the albumin peaks indicated in each of the patterns illustrated in Figure 8 was checked against that of pure serum albumin electrophoresed under the same conditions. The results of three experiments with different pairs of sera were virtually identical.

As is evident in Figure 8, the mobility of bilirubin was identical in both types of serum at all levels of pH except 5.0. Between pH 6.0 and 9.0 the two pigments migrated as well-defined bands which coincided with their respective albumin peaks. However, at pH 5.0 a marked difference was observed. In the direct-reacting serum the bilirubin was concentrated in the albumin fraction, but could also be detected in small amounts in the trail of the globulins. In contrast, the pigment in the indirect-reacting serum was confined to a narrow band close to the anodal side of the starting line, and had the granular appearance of a precipitate. Since the serum had been applied at the cathodal end of the strip, it appeared likely that the movement of the indirect-reacting pigment was due not to its linkage with a non-albuminous protein, but rather to capillarity and the siphoning effect known to occur as buffer evaporates from the center of the filter-paper strip during electrophoresis (16). In order to determine whether the pigments were bound to protein, duplicate sera were electrophoresed for 16 hours, following which one of the strips was removed for staining, while the other was subjected to an additional 7-hour period of electrophoresis with the poles reversed, which served to return the proteins toward their line of origin. It was found that the pigment in the indirect-reacting serum remained stationary despite the backward movement of the proteins, indicating that it was no longer linked to protein, while the pigment in the direct-reacting serum continued to migrate with the albumin peak indicating an intact bond. By streaking the serum over the center of the strip instead of at its cathodal end, it was possible to reduce the siphoning effect previously mentioned. As a result, the indirect-reacting bilirubin no longer migrated toward the anode, but appeared as a broad band

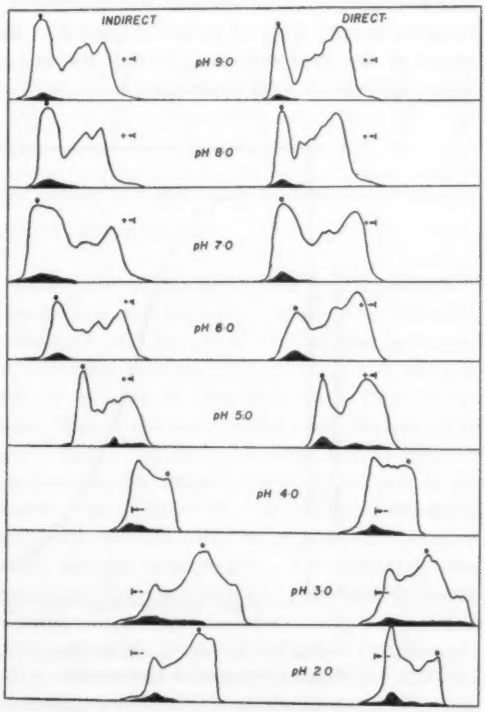

FIG. 8. EFFECT OF pH ON THE RELATIVE ELECTROPHORETIC MOBILITIES OF BILIRUBIN (SHADED AREAS) AND ALBUMIN (SOLID CIRCLES) IN DIRECT- AND INDIRECT-REACTING SERUM

The former was obtained from a patient with cirrhosis and contained 25.5 mg. of bilirubin per 100 ml., of which 11.7 mg. reacted directly; the latter was obtained from an infant with erythroblastosis fetalis and contained 10.4 mg. of bilirubin per 100 ml., of which 0.1 mg. reacted directly.

centered over the line of application. In contrast, the bulk of the direct-reacting bilirubin was still evident in the albumin fraction as it moved toward the anode; however, the previously noted pigment-trail in the globulin range was more prominent. These observations suggested that the bilirubin-albumin complex of serum was split at a pH of 5.0, releasing relatively insoluble free bilirubin, and that the cleavage was more complete in the case of indirect-reacting serum. Conceivably, the unbound pigment found in the globulin trail of the direct-reacting serum represented the indirect-reacting fraction present originally, while the pigment bound to albumin was all of the direct-reacting type.

At levels of pH between 2.0 and 4.0 it was found that the separation of bilirubin and albumin was complete in both types of serum (Figure 8). Reversal of the migration and shifting the line of serum application were again used to exclude the possibility that bilirubin was bound to a non-albuminous protein. At pH 2.0 and 3.0 there was distinct precipitation to the cathodal side of the starting line, not only of bilirubin but also of protein. Beyond this point both the protein and pigment were arranged in a pattern of irregular longitudinal streaks.

It is clear from these experiments that the electrophoretic mobilities of direct- and indirect-reacting bilirubin in serum are identical with that of albumin over a pH range of 6.0 to 9.0. Since albumin and globin differ markedly with respect to their isoelectric points, which lie at pH 4.4 (*27*) and 7.5 (*28*), respectively, the two proteins could hardly migrate as a single peak at pH 6.0 and 7.0. It must be concluded, therefore, that the bulk of the indirect-reacting bilirubin of icteric serum is not bound to globin.

The observation that both types of bilirubin separate from their albumin complexes when the

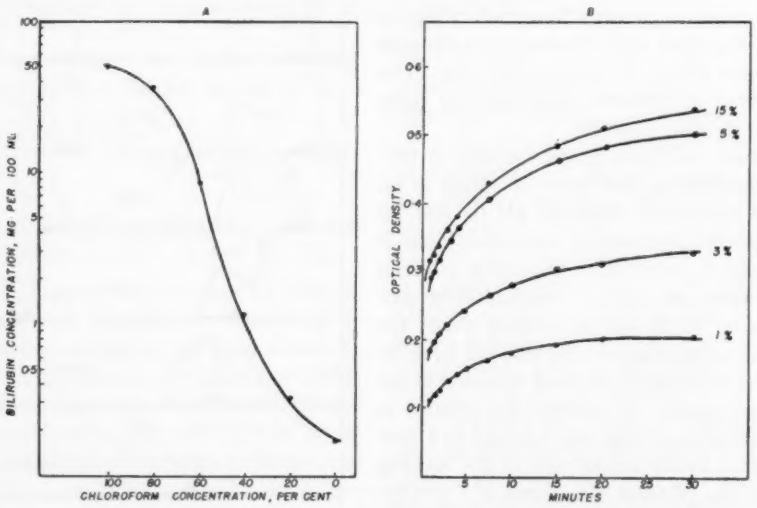

FIG. 9. A. SOLUBILITY OF PURE BILIRUBIN (HOFFMAN-LAROCHE) IN VARYING CONCENTRATIONS OF CHLOROFORM IN METHANOL

An excess of bilirubin was shaken for one-half hour with each of the mixtures in a sealed nitrogen-filled bottle. The concentration of pigment was determined spectrophotometrically at 450 mμ following centrifugation and filtration. Values shown are averages of triplicate determinations.

B. DIAZOTIZATION REACTION CURVES OF BILIRUBIN IN INCREASING CONCENTRATIONS OF CHLOROFORM IN METHANOL SHOWING THE DIRECT RELATIONSHIP BETWEEN SPEED OF DIAZOTIZATION AND CHLOROFORM CONCENTRATION

All reaction mixtures contained 10 ml. of water and 0.4 mg. of bilirubin per 100 ml. The remainder was made up of varying proportions of methanol and chloroform as indicated. Values shown are averages of triplicate determinations.

TABLE II

Recovery of direct- and indirect-reacting bilirubin in mixtures of serum of differing bilirubin content*

Type of serum	Initial concentration			Theoretical† concentration of mixture			Concentration found in mixture			Per cent of theoretical value recovered		
	Direct	Indirect	Total	Direct	Indirect	Total	Direct	Indirect	Total	Direct	Indirect	Total
	mg. per cent	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*			
Obstructive j.	17.04	17.04	34.08									
Normal	0.19	0.38	0.57									
Mixture 1 : 1				8.61	8.71	17.32	8.52	8.66	17.18	99	99	99
Obstructive j.	14.25	22.70	36.95									
Normal	0.19	0.76	0.95									
Mixture 1 : 1				7.22	11.74	18.96	7.22	11.02	18.24	100	94	96
Obstructive j.	18.00	16.37	34.37									
Hemolytic j.	0.82	11.09	11.91									
Mixture 1 : 1				9.41	13.73	23.14	9.22	14.40	23.62	98	105	102
Obstructive j.	5.90	2.98	8.88									
Hemolytic j.	0.48	4.94	5.42									
Mixture 1 : 1				3.19	3.96	7.15	3.31	3.91	7.22	104	99	101
Obstructive j.	5.57	3.45	9.02									
Hemolytic j.	0.31	5.02	5.33									
Mixture 1 : 1				2.95	4.22	7.17	3.05	4.18	7.23	103	99	101
Mixture 1 : 2				2.06	4.49	6.55	2.04	4.97	7.01	99	111	107
Mixture 1 : 3				1.63	4.63	6.26	1.87	4.56	6.43	114	98	103
Mixture 2 : 1				3.82	3.98	7.80	3.94	3.82	7.76	103	96	99
Mixture 3 : 1				4.27	3.84	8.11	4.13	4.22	8.35	97	110	103

* Determined by a modification (14) of the Malloy-Evelyn method (15) adapted to the Beckman DU spectrophotometer.

† Values expected if there were simple summation of two distinct forms of bilirubin.

pH falls below 5.0 is of considerable interest, since diazotization is usually carried out at a pH of 2.0 to 4.0, depending on the technique employed. Obviously, then, the direct and indirect reactions cannot be related to differences in protein linkage. An alternative possibility that merits consideration is that the type of reaction depends on the solubility of unbound bilirubin in an acid medium. At the pH of the van den Bergh reaction the pigment is known to be relatively insoluble in water (29) and, hence, incapable of diazotization (30). However, in the case of serum-diazo mixtures significant solution must occur, since a direct-reacting fraction of pigment can be detected in all sera (1, 14). It is conceivable, therefore, that the percentage of bilirubin that reacts directly is a function of solubility, dependent either on differences in the solvent properties of serum itself, or on the presence of variable proportions of two pigments with different solubilities at low pH. Accordingly, substances like alcohol and caffeine might facilitate the diazotization of indirect-reacting bilirubin by increasing its solubility.

In support of this hypothesis it was possible to demonstrate a relationship between the solubility of bilirubin and its rate of diazotization in several experimental systems. However, it was not possible to do so in the case of serum. Thus, it was found that in chloroform-methanol mixtures both the solubility and rate of diazotization of bilirubin increased as the proportion of chloroform in the system rose (Figure 9). A similar relationship could also be established in an aqueous system in which the pH was varied. The possibility was considered that the solubility of bilirubin in direct-reacting serum might be enhanced by the presence of the hypothetical catalyst described by Cantarow (31). However, when sera containing different proportions of direct- and indirect-reacting bilirubin were mixed, the final concentrations observed were those predicted for the simple summation of two pigments (Table II), and did not show the relative increase in the direct-reacting fraction reported by Cantarow (31). Similarly, when ultrafiltrates of normal and strongly direct-reacting serum were compared with respect to their capacity to enhance the diazotization rate of

a slow-reacting alkaline solution of bilirubin in albumin no difference was observed.

Although there is no evidence to support the hypothesis that the ratio of direct- to indirect-reacting bilirubin in serum depends on the solvent properties of serum itself, it is difficult to exclude the possibility that the ratio is related to the presence of varying proportions of two pigments with different solubilities. Indeed, the recent observations of Cole and Lathe (32), indicating that the two pigments can be separated by reverse phase chromatography, and that the direct-reacting type is more soluble in water, are consistent with this interpretation.

Najjar and Childs (33) have found that, when serum is brought to a pH of 5.0, ether will extract most indirect-reacting pigment, but only 2 per cent of the direct-reacting type, a phenomenon consistent with our observation that the former separates from its albumin complex at pH 5.0, and the latter at pH 4.0. It is also known that the two pigments differ with respect to their solubility in organic solvents at the normal pH of serum (6, 34). These observations suggest that the linkage between bilirubin and albumin differs in the two types of serum, and are also consistent with the concept that there is a subtle difference in the structure of the two pigments.

SUMMARY

By means of filter-paper electrophoresis it has been shown that most, if not all, of the bilirubin in serum is bound to albumin, irrespective of its behavior in the van den Bergh reaction. While it is not possible by this technique to exclude the presence of small amounts of pigment in the alpha$_1$-globulin of Cohn's Fraction V-1, it has been calculated, from what is known about its concentration in plasma and its bilirubin-binding capacity, that it cannot account for more than 0.05 mg. of bound pigment per 100 ml. of serum.

Several lines of evidence have been presented to show that indirect-reacting serum bilirubin is not a globin complex. These include the demonstration that (a) the electrophoretic mobility of native globin differs from that of serum bilirubin, (b) the direct- and indirect-reacting pigments cannot be separated electrophoretically at pH 6.0, a level at which albumin and globin migrate toward opposite poles, and (c) the solubility and bilirubin-binding capacity of globin are so low at the normal pH of the blood that a globin complex, if present, could not account for more than 1.0 mg. of bound pigment per 100 ml.

Attempts to distinguish between the two types of pigment in serum on the basis of their ammonium sulfate fractionation patterns have been unsuccessful, both patterns invariably coinciding with that of albumin.

It has been found that both types of bilirubin in serum remain firmly bound to albumin at levels of pH between 6.0 and 9.0, and separate at levels below 5.0. Only at pH 5.0 has a difference been observed, the separation of pigment from protein being relatively complete in the indirect-reacting type and partial in the direct-reacting, suggesting that the protein-linkages of the two pigments differ. Since the van den Bergh reaction is usually carried out at a pH of 2.0 to 4.0, it must be assumed that both types of bilirubin are in an unbound state when they react with the diazo reagent.

The implications of these observations have been discussed and the possibility has been considered that the variable behavior of bilirubin on diazotization may be related to differences in its solubility in an acid medium. Recent chromatographic evidence, suggesting that the two pigments differ with respect to their chemical structure and solubility in water, appears to be consistent with this hypothesis.

It is concluded that (a) the bulk of bilirubin in serum is bound to albumin, irrespective of its behavior in the van den Bergh reaction, (b) there is no evidence that, in the breakdown of hemoglobin, globin remains attached to its prosthetic group to yield indirect-reacting bilirubin, (c) the two types of van den Bergh reaction are not related to differences in bilirubin-protein linkage, and (d) the action of alcohol in facilitating the diazotization of indirect-reacting bilirubin does not depend on the cleavage of a pigment-protein complex.

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THE MISCIBLE POOL AND TURNOVER RATE OF HYDROCORTISONE IN MAN

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In 1948, Mason and Sprague (1) reported the isolation of hydrocortisone from the urine of a patient with Cushing's syndrome, and thus provided evidence that hydrocortisone was produced by the human adrenal cortex. Subsequently, hydrocortisone was found to be one of the principal corticoids in human adrenal gland perfusates (2) and in human peripheral blood (3). These observations strongly suggest that hydrocortisone may be the principal corticosteroid secreted by the adrenal cortex of man. Recent studies (4, 5) utilizing hydrocortisone-4-C^{14} have elucidated much new information regarding the metabolism and physiological disposition in man of this naturally occurring adrenal steroid.

The availability of labeled hydrocortisone has made possible the direct estimation of the magnitude of the reservoir of hydrocortisone in the body, and the rate at which new, non-isotopic hydrocortisone is synthesized in the body and enters the reservoir. This report is concerned with the experimental determination of the magnitude of the miscible pool of hydrocortisone and the rate of its turnover in man. These estimations depend upon serial measurements of the specific activity of circulating hydrocortisone after the infusion of trace quantities of hydrocortisone-4-C^{14} and analyses of these data utilizing conventional turnover calculations (6, 7). Observations have been made in normal subjects, and subjects receiving adrenocorticotropin and Δ^1 cortisone (prednisone).

MATERIALS AND METHODS

Nine normal subjects (7 male and 2 female) were used for these studies. Each subject received 1 to 2.5 microcuries of hydrocortisone-4-C^{14} dissolved in 2 to 5 ml. of 10 per cent ethanol in sterile distilled water. The steroid was administered intravenously in the fasting state in the morning over a period of approximately 3 minutes. Heparinized blood samples (40 to 60 ml. each) were collected at 30 to 40-minute intervals after injection of the isotope.

Procedure for determination of specific activity of circulating hydrocortisone

Twenty-five to 35 ml. of plasma were extracted gently for 10 minutes on a rotator (Arthur H. Thomas Co., Catalog No. 3623) in a 700-ml. Erlenmeyer flask with 5 volumes of dichloromethane (purified by passing through a column of silica gel [5]).

The plasma and solvent were gently transferred to a 200-ml. ground-glass stoppered cylinder, and the plasma removed by aspiration.

The dichloromethane extract was washed successively with 1/15 volume of 0.01 N sodium hydroxide, 1/15 volume of 0.1 M acetic acid, and 1/15 volume of water.

The washed extract was evaporated to dryness *in vacuo* at a temperature of 30 to 40° C.

The residue was dissolved in a small volume of dichloromethane and then applied to Whatman No. 1 filter paper (16 by 55 cm.) previously washed continuously with reagent grade methanol for 10 or more days in a large chromatography cabinet (Chromatocab).[1] The filter paper was kept in the Chromatocab until ready for use. A reference standard containing 5 to 10 micrograms of hydrocortisone was run on the same filter paper strip.

After an initial 1 to 2-hour equilibration period at room temperature in a modified Bush-type (8) system (benzene 4: methanol 2: water 1), the chromatogram was developed for 6 to 8 hours.

Following the chromatography, the strip was removed and scanned with ultraviolet light. The hydrocortisone standard and plasma hydrocortisone were located and marked with pencil (hydrocortisone moves approximately 10 cm. in 6 hours).

A small transverse band of paper (15 mm. wide and 30 mm. long) containing the plasma hydrocortisone was cut out. The band was cut as narrow as possible, perpendicular to the running direction of the solvent. When the plasma hydrocortisone was present in quantities insufficient to detect with the ultraviolet light, the band was cut at a position directly opposite the hydrocortisone standard.

The hydrocortisone was eluted from the paper with 3 to 4 ml. of cold 95 per cent redistilled ethanol, and the ethanol evaporated to dryness with nitrogen.

The residue was dissolved in a small volume of purified dichloromethane (4 to 8 ml.). An aliquot of known

[1] "Chromatocab." Research Equipment Corporation, Oakland, California.

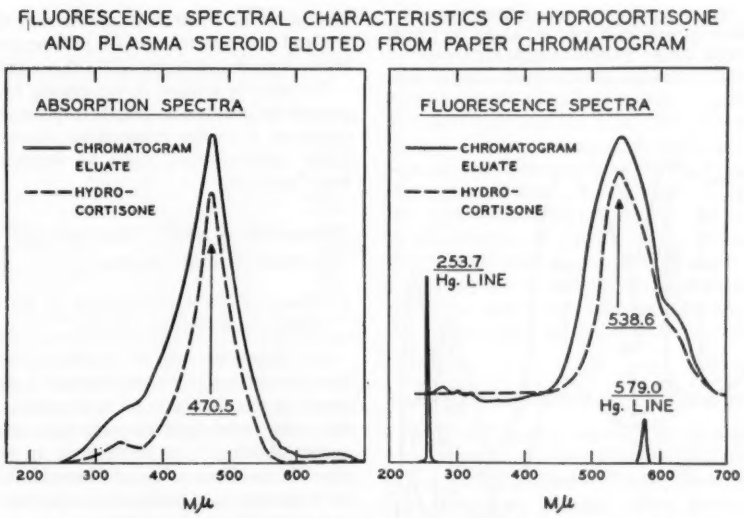

FIG. 1. FLUORESCENCE SPECTRAL CHARACTERISTICS OF HYDROCORTISONE AND THE PLASMA STEROID ELUTED FROM PAPER CHROMATOGRAM

Spectra determined in mixture 7 parts H_2SO_4 and 3 parts ethanol.

volume was transferred to a small conical ground-glass stoppered tube for fluorescence assay.

An aliquot for carbon[14] assay was evaporated to dryness and the residue dissolved in methanol and transferred quantitatively to a stainless steel planchet (surface area of 1.6 sq. cm. and depth of 0.6 cm.), and the methanol evaporated to dryness on a rotating platform with the aid of a stream of air and an infra-red lamp. The samples were counted in a Robinson gas-flow detector (9) to within an error of approximately 3 per

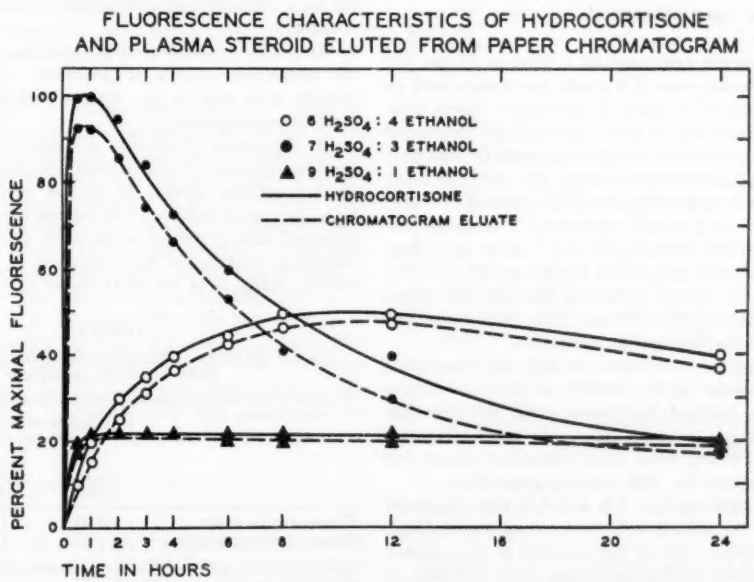
FLUORESCENCE CHARACTERISTICS OF HYDROCORTISONE AND PLASMA STEROID ELUTED FROM PAPER CHROMATOGRAM
PERCENT MAXIMAL FLUORESCENCE
0 20 40 60 80 100
6 H2SO4 : 4 ETHANOL
7 H2SO4 : 3 ETHANOL
9 H2SO4 : 1 ETHANOL
HYDROCORTISONE
CHROMATOGRAM ELUATE
0 1 2 3 4 6 8 12 16 20 24
TIME IN HOURS

FIG. 2. FLUORESCENCE CHARACTERISTICS OF HYDROCORTISONE AND THE PLASMA STEROID ELUTED FROM PAPER CHROMATOGRAM

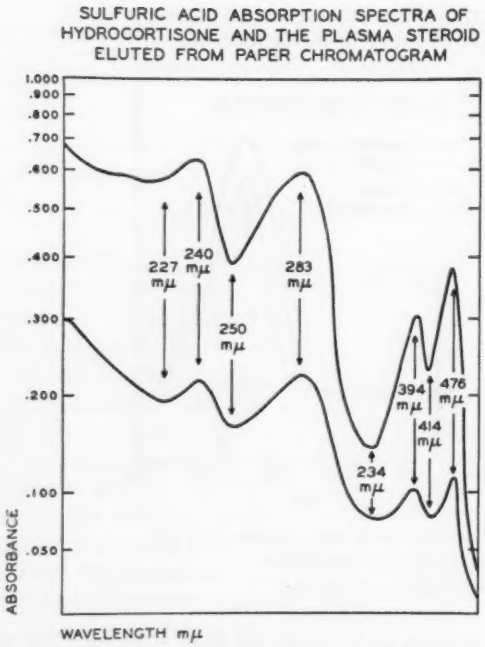

FIG. 3. SULFURIC ACID ABSORPTION SPECTRA OF HYDROCORTISONE (UPPER CURVE) AND THE PLASMA STEROID ELUTED FROM PAPER CHROMATOGRAM (LOWER CURVE)

Steroids incubated at room temperature for 2 hours in concentrated sulfuric acid and spectra run in Beckman DK Recording Spectrophotometer.

cent. This detector connected to a Nuclear Model 172 scaler gave a background of 3 counts per minute with an efficiency for carbon[14] of about 53 per cent at infinite thinness. All counting results were corrected for instrument variation by reference to a barium carbonate-C^{14} standard.

The other dichloromethane aliquot was used for assay of fluorescence by a modification of the method of Sweat (10). One ml. of a reagent containing 7 parts by volume of concentrated sulfuric acid and 3 parts by volume of redistilled ethanol was added *rapidly* to the dichloromethane, and the steroid extracted into the acid phase by vigorous shaking, *immediately*. The dichloromethane (upper) layer was removed by aspiration.

After standing for 60 minutes the acid was transferred to a 1 cm. diameter pyrex cuvette and the fluorescence measured in a Farrand fluorimeter using the following filter combinations: primary filter, 470 mμ interference and No. 5133 Corning glass filter; secondary filters, 540 mμ interference and No. 3486 Corning glass filter.

Standards containing 0.25, 0.5, and 0.75 micrograms of hydrocortisone were read with the unknowns. A linear relationship existed between the intensity of fluorescence and concentrations of hydrocortisone over a range of 0.01 to 2.0 micrograms. The plasma samples and hydrocortisone standards were read against a dichloromethane reagent blank. Filter paper blanks were also run, but with levels of hydrocortisone of 0.5 micrograms or more, these blanks contributed a negligible fluorescence.

The specific activity of the plasma hydrocortisone expressed as counts per minute per microgram was then calculated from the radioactivity value and the quantitative hydrocortisone value as determined by fluorescence analysis.

Evaluation of Basic Assumptions Necessary for Turnover Rate Studies

1) *Purity of the hydrocortisone of plasma extracts and paper chromatogram eluates*

(a) *Migration rate on chromatogram*—The dichloromethane extract of plasma contains a substance that migrates at the same rate as hydrocortisone, as determined with ultraviolet light scanning and alkaline blue tetrazolium staining. On addition of authentic hydrocortisone to the dichloromethane extract of plasma (mixed chromatogram), a single spot migrating at the same rate as hydrocortisone was found.

(b) *Fluorescence spectra*—Figure 1 shows the spectral curves of fluorescence (activating and emitting energies) of authentic hydrocortisone and of the substance eluted from paper after chromatography of a plasma dichloromethane extract.[2] Figure 2 shows the fluorescence intensities of authentic hydrocortisone and the substance present in the chromatogram eluate as they vary with time in various concentrations of sulfuric acid and ethanol. The fluorescence intensity-time curves at these three concentrations of sulfuric acid appear to be characteristic for each individual steroid which fluoresces under these conditions.

(c) *Sulfuric acid absorption spectra*—Figure 3 shows the absorption spectra of authentic hydrocortisone in sulfuric acid, and of the substance eluated from paper after chromatography.

(d) *Constancy of specific activity*—Table I lists the specific activities of the steroid (hydrocortisone) in a dichloromethane extract of plasma eluted from paper after chromatography. (The plasma consisted of a pool collected from 3 normal subjects 30 minutes to 2 hours following intravenous administration of hydrocortisone-4-C^{14}.) Aliquots of the paper eluate were assayed by the

TABLE I

Specific activities of plasma hydrocortisone after elution from paper chromatogram

	Counts per minute/microgram		
	Hydrocortisone	Hydrocortisone acetate	Tetrahydrohydrocortisone
Fluorescence assay	130	125	
Phenylhydrazine assay	140	140	125

[2] We are indebted to Dr. Robert Bowman, of the National Heart Institute, for the spectral studies.

TABLE II

Partition coefficients of authentic hydrocortisone and labeled plasma hydrocortisone after elution from paper chromatogram

Solvent systems	K* authentic hydrocortisone	K plasma hydrocortisone	Ratio specific activity upper and lower solvent phases
Water/CH_2Cl_2(1) : CCl_4(1)	0.91	0.91	1.05
Water/CH_2Cl_2(1) : CCl_4(4)	5.40	6.00	1.08
Benzene/water	0.60	0.66	0.90

* K = Partition coefficient (upper phase/lower phase).

fluorescence method, the phenylhydrazine method (5, 11), and by radioactive counting. In addition, aliquots of the eluted steroid were evaporated to dryness and the residue acetylated. The acetylated product was rechromatographed in the benzene-methanol-water system, eluted, and its specific activity determined. An additional aliquot was reduced enzymatically (12) to the tetrahydro derivative, rechromatographed in the benzene-methanol-water system, and the specific activity again determined. Finally, additional aliquots were taken for a study of partition coefficients (Table II). The eluted steroid was partitioned between 3 different solvent systems, and the ratios of specific activities in the 2 solvent phases determined. A deviation from a ratio of 1.0 would have constituted evidence for non-identity of the plasma steroid with hydrocortisone.

2) *Steady state* (Constancy of the magnitude of the rapidly exchangeable pool, and of the quantities of hydrocortisone entering and leaving this pool per unit time.)

Determinations of plasma hydrocortisone concentrations were made at the beginning and at the end of the study, and in none of these subjects did a variation in the plasma level of more than ± 15 per cent occur. The subjects were therefore considered to be in a steady state with regard to plasma hydrocortisone concentrations, and thus also with regard to the magnitude of the miscible pool. It was therefore assumed that the rate of entry of hydrocortisone into the pool equalled the rate of its disappearance from the pool. The fact that after 20 to 30 minutes following injection of the hydrocortisone-4-C^{14} the experimental specific activity values were distributed close to a straight line on a semilogarithmic plot *vs.* time (Figure 4) is in conformity with this assumption regarding the steady state.

3) *Failure of isotopic hydrocortisone to re-enter the miscible pool*

It appears likely that hydrocortisone, once removed from the circulating pool by the liver, does not again re-enter the pool as unaltered hydrocortisone. This conclusion is strongly suggested by an experiment wherein specific activities of circulating hydrocortisone, sampled simultaneously from a peripheral vein and from the hepatic vein by catheterization, were compared following intravenous administration of a trace quantity of labeled hydrocortisone (Figure 5). The specific activities of hydrocortisone obtained from both sites were similar for at least 3 hours, indicating no significant reversible concentration of hydrocortisone by the liver. Furthermore, although metabolites of hydrocortisone do appear in the bile (5), no unaltered hydrocortisone is thus excreted, so that re-entry of the steroid into the miscible pool by intestinal absorption appears excluded. Finally, the plasma protein binding of hydrocortisone (5) is readily reversible, as dialysis studies have shown, thus dismissing the consideration of inhomogeneity within the pool on this basis.

4) *Rapid mixing within the circulating pool*

When the specific activities of circulating hydrocortisone determined serially after the intravenous administration of hydrocortisone-4-C^{14} were plotted on semilogarithmic coordinates, a rapid initial decline was observed (Figure 4). However, after 20 to 30 minutes, a single

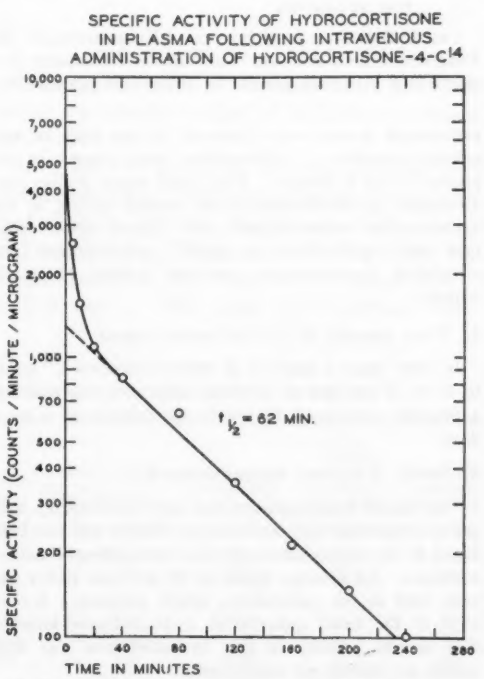

FIG. 4. SPECIFIC ACTIVITY OF HYDROCORTISONE IN PLASMA FOLLOWING INTRAVENOUS ADMINISTRATION OF HYDROCORTISONE-4-C^{14}

The data of this experiment (Subject J. B., Table III), when analyzed by the method of least squares, indicated a miscible pool of 1.052 ± 0.100 mg. and a turnover rate of 0.709 ± 0.087 mg. per hour.

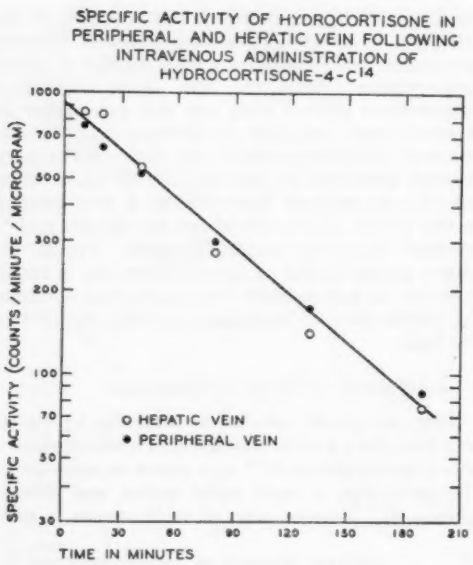

FIG. 5. SPECIFIC ACTIVITY OF HYDROCORTISONE IN PERIPHERAL AND HEPATIC VEIN BLOOD FOLLOWING INTRAVENOUS ADMINISTRATION OF HYDROCORTISONE-4-C^{14}

exponential process was observed for as long as significant quantities of radioactivity were present in the plasma (4 to 6 hours). The initial rapid decline was attributed to distribution of the labeled steroid in the exchangeable hydrocortisone pool. These results suggest that equilibration of labeled hydrocortisone and circulating hydrocortisone occurred within 20 to 30 minutes.

5) *Trace quantity of injected hydrocortisone*

In most cases a quantity of hydrocortisone-4-C^{14} equal to 10 to 25 per cent of the total body pool was injected, a quantity considered to satisfy the criteria of a trace dose.

6) *Purity of injected hydrocortisone-4-C^{14}*

The labeled hydrocortisone has been evaluated by both paper chromatography and isotope dilution and has been found to be approximately 94 per cent authentic hydrocortisone. An average figure of 94 per cent purity has been used in the calculations, which requires a knowledge of the exact quantity of hydrocortisone injected. The specific activity of this hydrocortisone was 4600 counts per minute per microgram.

RESULTS

After the injection of hydrocortisone-4-C^{14}, a rapid initial fall in specific activity was observed (Figure 4). After approximately 20 minutes, a more gradual decline occurred. This gradual decline represented a first order function, since a semilogarithmic plot revealed that these points approximated a straight line. This exponential decay was interpreted as a measure of the rate of replacement of the labeled hydrocortisone by newly synthesized unlabeled hydrocortisone.

By extrapolation of the linear portion of the curve of specific activity to zero time, it was possible to determine the specific activity of the hydrocortisone in the body (miscible pool) capable of prompt mixing, immediately after injection (6). From this value (I_o), and knowledge of the quantity (a) and specific activity (I_i) of the injected tracer, the magnitude of the miscible pool (A) was calculated.

$$A = a\left(\frac{I_i}{I_o} - 1\right)$$

The biological half-time of the hydrocortisone was obtained graphically from the linear plot of the slow component.[3] The negative slope of the line gave the turnover rate expressed in pools per unit time, which was computed from the following expression (7):

$$\text{Turnover rate (K)} = \frac{\log 2}{t_{\frac{1}{2}}}$$

This expression is derived from the equation for a first order reaction:

$$I = I_o e^{-Kt}$$

where

I = hydrocortisone-4-C^{14} at any time
I_o = hydrocortisone-4-C^{14} at zero time
t = time in hours
K = fraction of all the hydrocortisone in the miscible pool which is replaced by non-isotopic hydrocortisone each hour.

Hydrocortisone turnover (KA), expressed in micrograms per hour, was computed from the value for the miscible pool (A) and the rate of its turnover (K) in pools per hour. This figure was projected to mg. turned over per day by multiplying by 24 and dividing by 1000.

[3] This biological half-time must not be confused with the previously reported biological half-time for the rate of disappearance of hydrocortisone from plasma (5). The decline in specific activity is a measure of the rate of *appearance* of newly synthesized unlabeled hydrocortisone into the pool, and is in no way related to the rate of disappearance of hydrocortisone from the pool.

TABLE III

Miscible pool of hydrocortisone and its turnover in normal subjects

Subject	Age	Wt. kilos (W)	Dose injected (a)	Intercept (I_0)	Miscible pool (A)	$t_{\frac{1}{2}}$	Turnover (K)	Turnover (KA)	Turnover (KA × 24)	Urinary corticoids§ (U)	Hydrocortisone turnover as urinary corticoids	Plasma hydrocortisone§ (P)	Volume of distribution [A (μg.)/10P]
			μg.	cpm/μg.	mg.	hours	Pool/hr.	mg./hr.	mg./day	mg./day	% (U/KA × 24)	μg. %	liters
T. R.	70	70	500	860	2.2	1.83	0.38	0.83	20	4.8	24.0	16	14
T. R.*	70	70	500	800	2.4	1.53	0.45	1.08	26	4.8	18.5	18	13
J. W.	30	73	500	1,200	1.4	1.23	0.56	0.80	19			11	11
R. T.	16	68	500	1,300	1.8	1.33	0.52	0.92	22	5.2	23.5	19	9.5
R. T.*	16	68	350	820	1.6	1.03	0.67	1.09	26	5.0	19.0	19	8.5
E. H.	16	67	500	930	2.0	1.43	0.49	0.96	23	4.3	19.0	16	12
E. H.*	16	67	350	700	2.0	1.38	0.50	0.98	24	4.8	20.0	16	12
E. P.†	20	67	300	520	2.4	2.30	0.30	0.70	17	4.0	23.5	24	10
L. C.†	23	66	300	920	1.2	1.08	0.64	0.77	19	5.0	26.0	15	8.0
J. B.	22	69	450	1,350	1.1	1.03	0.67	0.74	18	7.0	39.0	13	8.5
W. R.	22	69	400	990	1.5	1.33	0.52	0.75	18	6.5	36.0	17	8.5
D. B.	26	84	270	460	2.4	1.30	0.50	1.22	29	10.5	38.0	14	17
D. B.‡	26	84	270	1,400	0.6	0.83	0.84	0.51	12			4	15

* The second experiments were done approximately 2 weeks following the first experiment.
† Women.
‡ The second study was started at 9:00 p.m.
§ Determined by method of Peterson and his co-workers (5, 11).

In order to evaluate the precision of the graphic determinations of the intercept, I_0, and of the rate constant, K, these values were also determined in several studies by the method of least squares. The probable error of pool size, $\pm \Delta A$, was determined by calculations previously published (13) and that of the rate constant, $\pm \Delta K$, by the method of least squares. The hydrocortisone pool size and daily turnover rate values determined by these two procedures agreed in all cases within 5 per cent, so that the graphic method of analysis was chosen for routine use.

In Table III are listed the data obtained in 9 normal subjects. The mean value for the miscible pool of hydrocortisone was 1.8 mg., with a range of 1.1 to 2.4 mg. The mean half-time of the hydrocortisone was 1.38 hours with a range of 1.03 to 2.30 hours. The mean hydrocortisone turn-

TABLE IV

Miscible pool of hydrocortisone and its turnover after suppression and stimulation of the adrenal

Subject*		Dose injected (a)	Intercept (I_0)	Miscible pool (A)	$t_{\frac{1}{2}}$	Turnover (K)	Turnover (KA)	Turnover (KA × 24)	Urinary corticoids†‡ (U′)	Hydrocortisone turnover as urinary corticoids†	Plasma hydrocortisone‡,§
		μg.	cpm/μg.	mg.	hours	Pools/hr.	mg./hr.	mg./day	mg./day	% (U′/KA × 24)	μg. %
R. U. 70 M 69 K	I	200	3,000	0.12	1.67	0.41	0.05	1.2	0.8	67.0	3
	II	500	220	10.00	1.08	0.64	6.42	154	46.0	30.0	44
L. B. 21 M 74 K	I	360	220	7.20	1.03	0.67	4.84	116	36.0	31.0	28
	II	360	200	7.90	0.95	0.73	5.53	133	38.0	28.5	30
	III	360	160	10.00	1.28	0.54	5.41	130	36.0	27.5	25

* R. U. (I) 8 hours after oral administration of 60 mg. prednisone over previous period of 42 hours.
R. U. (II) 6 hours after start of continuous infusion of 5 units adrenocorticotropin per hour.
L. B. 6 hours (I), 18 hours (II), and 40 hours (III) after start of continuous infusion of 5 units adrenocorticotropin per hour.
† Urinary excretion based on 2-hour urinary excretion at 6 to 8 hours (R. U. II; L. B. I), 18 to 20 hours (L. B. II), and 40 to 42 hours (L. B. III).
‡ Determined by method of Peterson and his co-workers (5, 11).
§ Mean plasma steroid value based on average of 6 different determinations made throughout the 3-hour period of the study.

over rate was 0.52 pool per hour, or 0.91 mg. per hour with a range of 0.70 to 1.22 mg. per hour.

In two of three subjects in whom a second experiment was performed some weeks after the first, moderate variations were found in both pool size and turnover rate. Whether these differences represent experimental errors or variations in the rate of production of hydrocortisone in these subjects is uncertain. However, in one subject (D. B.) turnover studies conducted at 9:00 a.m. and at 9:00 p.m. (four days later) revealed clearly that hydrocortisone production diminished late in the evening compared with the morning. In this subject the hydrocortisone pool was 2.4 mg. at 9:00 a.m. and 0.6 mg. at 9:00 p.m. Corresponding plasma hydrocortisone concentrations were 14 and 4 micrograms per cent. Hydrocortisone production was estimated to be 1.22 mg. per hour in the morning and 0.51 mg. per hour in the late evening.

In Table IV are listed the turnover rate data in two normal male subjects obtained after the continuous intravenous administration of 5 units of adrenocorticotropin per hour over periods of 10 hours (R. U.) and 44 hours (L. B.). In R. U. the turnover study was started 6 hours after the beginning of the infusion and in case L. B. turnover studies were started at 6, 18, and 40 hours. Hydrocortisone pool sizes ranging from 7.2 to 10.0 mg. were found, and hydrocortisone production values equivalent to 116 to 154 mg. per day were calculated. In patient R. U., a turnover study was also done after the suppression of adrenal activity with orally administered Δ^1 cortisone (prednisone) (10 mg. every 6 hours—first dose given 42 hours before start of study and turnover study started 8 hours after last dose). Endogenous hydrocortisone production was reduced to a rate of 1.2 mg. per day.

As an adjunct to the above results, estimations were made of the apparent volumes of distribution of the hydrocortisone pool. These were obtained by dividing the size of the pool in micrograms by the concentration of hydrocortisone in plasma in micrograms per ml. (The method used for the determination of plasma hydrocortisone has been shown to be specific for hydrocortisone [11].) Values of 8 to 17 liters were obtained for the apparent volume of distribution of hydrocortisone.

DISCUSSION

Implicit in all isotopic turnover studies are certain assumptions, the validity of which must be evaluated in each case. An important consideration is that hydrocortisone only is being measured, and that in the determination of radioactivity only that radioactivity is measured that is due to unchanged hydrocortisone. The method described by Peterson and his co-workers (5, 11) has been shown to be specific for hydrocortisone normally present in plasma dichloromethane extracts, and this method would therefore be adequate for determining the hydrocortisone concentrations in plasma. However, a direct dichloromethane extract of plasma samples drawn after the infusion of hydrocortisone-4-C^{14} contains many free steroids that are radioactive but are not hydrocortisone. These metabolites may account for up to 50 per cent of the radioactivity in the dichloromethane extracts of plasma (5). Data are presented to show that the steroid eluted from paper and measured fluorometrically is indeed hydrocortisone-4-C^{14}. It may be of interest to note that the methods of analysis used do not depend upon obtaining quantitative recoveries from either the plasma extractions or the paper chromatography.

Another fundamental assumption is that the subject is in a steady state throughout the duration of the study, *i.e.*, that the total quantity of the compound whose turnover is being measured is sensibly constant, and therefore that the rate of addition of newly formed compound to the miscible pool is balanced by a similar rate of removal of compound from this pool. Further, it is assumed that mixing of the fraction added within the pool is rapid with respect to the frequency of sampling, and therefore that the samples withdrawn faithfully reflect the characteristics of the entire pool.

The validity of these assumptions has been carefully examined in the present study. As was pointed out, the level of phenylhydrazine reactive material extracted from plasma was essentially constant during the 3-hour period of study in all subjects tested. Other studies have demonstrated that this plasma component consists very predominantly of hydrocortisone (5, 11), so that the existence of a steady state during the short period of study may logically be inferred. It must be pointed out, however, that diurnal variations in

plasma hydrocortisone levels (14) and in urinary corticoid excretion (15) do occur, and indeed were demonstrated in some subjects of the present study. Such variations, when noted, indicated maximal adrenal activity during mid-morning hours, relative stability during the forenoon, a gradual decline in activity throughout the afternoon and evening, and minimal adrenal activity in the late evening and early morning hours. In one subject studied both in the mid-morning and late evening, a rather striking reduction in the rate of synthesis of hydrocortisone in the late evening hours was demonstrated. Since, with this one exception, the turnover studies reported in this paper were conducted in the forenoon hours, after the period of accelerated adrenal activity and before the period of tapering decline, it appears likely that these measurements of hydrocortisone turnover not only reflect steady state conditions but also constitute adrenal cortical secretion values which may be considered representative samplings of the average cortical activity of the entire day. For these reasons it appears valid to express hydrocortisone turnover in terms of daily production, even though the results were derived from a short sampling period.

In the normal individuals, the miscible pool of hydrocortisone averaged 1.8 mg., with a range of 1.1 to 2.4 mg. Approximately one-half this pool was replaced by newly synthesized hydrocortisone each hour during the period of the study. In these subjects the projected daily turnover of hydrocortisone averaged 22 mg., with a range of 17 to 29 mg.

The direct measurement of hydrocortisone production, as reported herein, has yielded values which confirm various estimates based on indirect methods, and in so doing establish the general validity of these approaches. For example, Dorfman (16) estimated hydrocortisone production to be 21 mg. per day, on the basis of the quantities of 11 oxy- and 11 desoxy-C^{19} neutral 17-ketosteroids in normal urine. Silber (17) also estimated daily production to 21 mg. per day, as judged from the quantity of endogenously produced phenylhydrazine chromogenes in urine, the calculations in each patient being based on the fraction of a test dose of cortisone excreted as similarly reactive metabolites. Bondy and Altrock (18) also arrived at a value of 21 mg. per day on the basis of renal vein catheterization studies. However, the apparent agreement of this value may be fortuitous since the plasma hydrocortisone method employed gave low values in normals, and the stress of the procedure might have been expected to alter adrenal activity.[4] Finally, clinical experience in Addisonian and adrenalectomized patients also indicates that hydrocortisone production in healthy individuals during average activity is in the range of 25 to 35 mg. per day (19). Of course, a patient with total adrenal insufficiency is also deficient in other cortical steroids such as corticosterone, aldosterone, and various C^{19} steroids, and the absence of these steroids may influence the maintenance requirements of hydrocortisone.

The method reported in this paper has provided the first direct information on the extent of the maximal adrenal response in normal subjects to the continuous intravenous administration of adrenocorticotropin. It has also demonstrated that after about 6 hours the adrenal response is maximal and that hydrocortisone synthesis does not increase during the next 36 hours despite continued administration of adrenocorticotropin. These results are in agreement with our data and the data of others on the plasma hydrocortisone and urine phenylhydrazine reacting steroid levels, after adrenocorticotropin stimulation. The plasma hydrocortisone concentrations reach a constant level after 4 to 6 hours in normals. Also, after 6 to 8 hours the urinary steroid excretion per 2-hour period remains constant for at least 44 hours.

After administration of a small quantity of prednisone, over a short period of time, a very marked suppression of adrenal hydrocortisone secretion results, thus demonstrating the great dependence of the adrenal for pituitary adrenocorticotropin. After this marked and rapid suppression, the adrenal in this normal subject was, however, capable of responding at a normal rate to exogenous intravenous adrenocorticotropin.

Previous studies (5) on the metabolic fate and physiological disposition of hydrocortisone have demonstrated that approximately 90 per cent of the radio-metabolites of either a tracer or a larger intravenous dose of this hormone are excreted in

[4] In the study conducted during hepatic catheterization, a projected hydrocortisone turnover of 40 mg. was found, and this high value in a normal subject was regarded as a manifestation of stress.

the urine. Less than one per cent of this quantity consists of unaltered hydrocortisone. After β-glucuronidase hydrolysis of conjugated metabolites, 20 to 30 per cent of the administered dose is recovered as phenylhydrazine reactive products, whereas 60 to 70 per cent of the administered dose is recovered by radioactive assay. The phenylhydrazine reactive group was discussed in terms of metabolites having reduced Δ_4, 3-keto groups, and the latter in terms of similarly reduced steroids having in addition α or β-20-ol groups (5), so as no longer to react with phenylhydrazine. These recent studies show clearly the reason for the great disparity between the values for hydrocortisone turnover (Table III, Column 10) and for urinary corticoids (Table III, Column 11) as shown in this paper. The lack of a very consistent relationship between the two values (see Table III, Column 12) illustrates the hazard of placing too great emphasis on urinary corticoid values as an accurate index of adrenal cortical function.

A similar caution may be interjected with reference to the level of hydrocortisone in plasma, particularly at rather low levels which may be compatible with turnover values well within the normal range. In addition, the plasma level is often normal in the presence of either increased or decreased adrenal hydrocortisone production. In cirrhosis, for example, plasma steroid levels are normal whereas urinary corticoids are low (20), and infused hydrocortisone disappears at a rate slower than normal (5). The hydrocortisone pool is normal (in the absence of ascites) but its turnover may be decidedly reduced (20). Again in thyrotoxicosis, plasma hydrocortisone levels are normal or low, urinary corticoids are normal or increased, and infused hydrocortisone disappears at a rate faster than normal (20). In this condition, the hydrocortisone pool is normal or low but its turnover is markedly increased. Similar studies in myxedema have disclosed a reduced turnover, in the presence of normal plasma steroid levels.

The apparent volume of distribution of the miscible pool of hydrocortisone has been determined in each patient of this study. The mean value averaged 11 liters with a range of 8 to 17 liters. It is unlikely that the volume has any physical reality, since protein binding in the extravascular spaces is probably not quantitatively identical with that in plasma, where 75 per cent of hydrocortisone exists in association with non-diffusible elements (5). This volume must therefore be regarded solely as that theoretical volume in which the miscible hydrocortisone exists at a concentration equal to that of plasma.

SUMMARY

Utilizing trace quantities of intravenously administered hydrocortisone-4-C^{14}, we have determined the magnitude of the miscible pool of hydrocortisone in the normal subject to be 1.1 to 2.4 mg. This pool of hydrocortisone has been found to be distributed in an apparent volume of 8 to 17 liters.

Calculations of turnover rate of endogenously synthesized hydrocortisone have ranged from 17 to 29 mg. per day in 9 normal subjects.

Following maximal adrenocorticotropin stimulation, the miscible pool of hydrocortisone increased to 10 mg., and the turnover rate increased to 154 mg. per day in the normal subject. Following oral administration of Δ^1 cortisone (prednisone), a marked decrease in both pool size and hydrocortisone turnover rate was demonstrated.

The miscible pool and turnover rate of hydrocortisone has been found to show a diurnal variation.

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ERYTHROCYTE PRESERVATION. VI. THE STORAGE OF BLOOD WITH PURINE NUCLEOSIDES [1]

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The addition of adenosine to stored blood results in a chemical restoration of deteriorated erythrocytes and sustains their viability during continued storage (1, 2). This phenomenon has been referred to as the *in vitro* reversibility of a biochemical lesion of storage. Apart from the implications of this reaction in red cell metabolism (3), it seemed of importance to evaluate the effect of adenosine in the preservation of blood. The present study indicates that adenosine and some related purine nucleosides retard the onset of the storage lesion and extend significantly the period of viability of stored blood. The effective concentration of these supplements, as well as their potential toxicity, is discussed.

METHODS

The techniques for the measurements of erythrocyte phosphate partition, blood glucose, sodium and potassium, osmotic fragility, and aerobic utilization of glucose have been described (1, 4). Oxygen content and oxygen capacity of erythrocytes were determined by the method of Van Slyke and Neill (5).

Isotonic saline solutions of adenosine and its analogs were employed in concentrations of 50 to 100 μmoles per ml. The nucleosides are soluble in warm saline. While relatively unstable with respect to re-precipitation at these concentrations, the nucleosides can be redissolved by subsequent heating. Sterilization of the solutions was accomplished by filtration through an ultrafine fritted pyrex glass disc. The method for the determination of adenosine concentration and absorption [2] has been reported (1).

In the experiments reported, the control sample refers to blood stored in ACD [3] alone. On the basis of *in vitro* studies on the synthesis of organic phosphate esters in stored blood, it was found unnecessary to maintain similar volumes in the samples within one experiment, and there were no additions made to the control aliquots, although nucleoside solutions were added to the experimental aliquots.

Post transfusion survival of stored red cells was measured with a double isotope technique in which Cr^{51} was employed as a tag for stored cells, and P^{32}-labeled fresh cells were used as a blood volume reference (6). When the measurements were performed in duplicate, the accuracy of the survival determination was considered to be ± 5 per cent. Theoretical survival was determined from the ratio of the two isotopes. Subsequent survival of the Cr^{51}-tagged cells was determined over a period of seven days. An excess loss of Cr^{51} of about 10 per cent occurs during the first 24 hours when washed fresh cells are transfused. The reported survival data are thus somewhat lower than would be expected from comparable studies employing the technique of differential agglutination of erythrocytes (Ashby). Before transfusion, cells stored with nucleosides, as well as control cells, were washed three times with saline. Hemolysis incident to washing was not usually significant before the 60th day of storage.

Toxicity measurements were carried out in rabbits and dogs by the intravenous administration of sterile saline solutions of the purine nucleosides and related products. In addition, plasma, and hemolyzed and fresh red cells from blood stored in ACD plus adenosine, were tested. Rabbits were anesthetized with ether, and dogs were given sodium pentobarbital intravenously. The blood pressure, pulse pressure, and heart rate were recorded directly from the cannulated femoral artery by means of a Sanborn Electromanometer Model No. 120 and a Sanborn Poly-Viso Recorder. A simultaneous electrocardiogram was obtained.

RESULTS

I. *Adenosine as a supplement to ACD in blood preservation*

A. *Rabbit blood.* The effects of a single initial supplement of adenosine compared with intermittent addition of this nucleoside during storage are illustrated by the following typical experiment. Blood, collected in ACD, was divided into three parts. Aliquot 1 served as a control. To Aliquot

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[2] Although this phenomenon could be absorption into the cell and/or adsorption onto the membrane, we shall use the term, absorption, throughout.

[3] National Institutes of Health, Formula B.

TABLE I

*Storage of rabbit blood in ACD+adenosine at 4°C.**

Days storage	μmoles P/100 ml. RBC		Cumulative absorption of adenosine (%)	Survival of RBC (%)
	Inorganic	Organic		
0	142	2,442		
15				
C-1	1,130	1,036		74
A-2	184	2,353	58	95
A-3	174	2,443	62	86
22				
C-1	1,317	601		68
A-2	292	2,220	66	87
A-3	180	2,454	66	88
29				
C-1	1,400	519		53
A-2	459	1,997	77	86
A-3	188	2,541	71	86
36				
C-1	1,470	362		38
A-2	615	1,641	81	82
A-3	199	2,563	74	83
43				
C-1	1,497	348		20
A-2	775	1,523	86	66
A-3	151	2,278	77	76
50				
C-1	1,500	289		7
A-2	863	1,153	90	66
A-3	174	2,581	80	81

* Blood was collected in ACD and divided into three aliquots: 1) Control (C-1); 2) Initial addition of 2,800 μmoles adenosine per 100 ml. RBC (A-2); 3) Addition of 1,400 μmoles adenosine per 100 ml. RBC weekly (A-3).

2 was added 2800 μmoles adenosine per 100 ml. erythrocytes (RBC) at the beginning of storage. To Aliquot 3, 1425 μmoles adenosine per 100 ml. RBC was added initially and the same amount each week throughout the storage period of 50 days. As shown in Table I, the per cent absorption [2] of adenosine was approximately the same in the treated samples, although the absolute amount taken up by the cells at 50 days in Aliquot 3 (7300 μmoles per 100 ml. RBC) was almost threefold greater than the uptake in Aliquot 2 (2590 μmoles). Higher levels of cellular organic phosphates were maintained in Aliquot 3, suggesting an increased utilization for this purpose. Erythrocytes of both treated samples retained their organic phosphate components (adenosine triphosphate, hexose phosphate, and 2,3-diphosphoglycerate) considerably better than did the control.

The correlated post-transfusion survival measurements (Table I) showed that at 15 days of storage, the erythrocytes stored in ACD alone were 74 per cent viable, while Aliquot 2 had 95 per cent survival, and Aliquot 3, 86 per cent. At this time the amount of adenosine available to the cells in the latter two samples was the same (2800 μmoles). This amount had been available to Aliquot 2 from the beginning of storage, while only 1400 μmoles was present in Aliquot 1 for the first week. The expected decline in survival of the control cells occurred subsequently. Both adenosine-supplemented samples showed about 85 per cent survival through 36 days. Thereafter, the viability of the erythrocytes of Aliquot 3 was better than that of Aliquot 2. Apparently the initial addition of adenosine, at the level employed, was adequate to maintain the red cells throughout the first five weeks of storage, and the weekly additions were not of additional benefit.

In Table II are shown the results of a long-term storage experiment in which rabbit blood was supplemented intermittently with 2300 to 2500 μmoles adenosine per 100 ml. RBC. Approximately 70 per cent of the adenosine was absorbed. A sediment, noted in the treated blood sample at 105 days of storage, was presumed to represent precipitated adenosine or its degradation product. This did not appear to affect the phosphate partition or the survival of these cells. The improved maintenance of the energy potential of

TABLE II

Storage of rabbit blood in ACD+adenosine at 4°C. for 105 days *

Days storage	μmoles Organic P/100 ml. RBC	Survival of RBC (%)
0	2,504	
25		
Control	847	44
Adenosine	2,000	65
54		
Control	408	5
Adenosine	1,417	56
77		
Adenosine	1,431	59
105		
Adenosine	1,271	44

* Blood was collected in ACD and divided into two aliquots: 1) Control; 2) Adenosine (2,300 to 2,500 μmoles per 100 ml. RBC) added on days 1, 25, 54, and 77.

TABLE III

*Post-transfusion survival of human blood stored in ACD+adenosine at 4°C.**

Days storage	Survival of RBC (%)
19	
Control	77
Adenosine	91
42	
Control	29
Adenosine	65
68	
Control	4
Adenosine	53

* Human blood was collected in ACD and divided into two aliquots: 1) Control; 2) 2,500 μmoles adenosine per 100 ml. RBC added at days 1, 19, and 42.

the cells, as indicated by organic phosphate levels, is evident in the adenosine-treated cells throughout storage. Prolongation of viability was demonstrated in the treated blood as compared with the control cells which were completely non-viable by the 50th day of storage.

B. *Human blood.* Comparable experiments were performed with human blood. In a typical study, 2500 μmoles adenosine per 100 ml. RBC was added every three weeks for approximately 10 weeks. The increased survival time of the adenosine-treated cells over a period of 68 days is shown in Table III. Other data which included phosphate partitions, osmotic fragilities, and determinations of the sodium-potassium gradient also indicated improved chemical characteristics of the erythrocyte when compared with the control blood stored in ACD alone.

C. *Miscellaneous studies.* Morphologic studies of red cells stored in ACD compared with those stored with the addition of adenosine showed some difference, but this could not be expressed quantitatively. The progressive spherocytosis, poikilocytosis, and crenation of stored blood was somewhat retarded by the addition of adenosine. Likewise, the flickering phenomenon, thought to bear some relation to normal function of the red cell (7), was maintained over a longer period of time. However, it was impossible to predict viability from these changes. The alterations in cell appearance, observed after 60 to 120 days of storage, were still compatible with appreciable cell survival (Tables II, III), which suggests that many of these morphologic changes are reversible upon re-entrance of the erythrocyte into active circulation. Appreciable spontaneous hemolysis was not observed before the 50th day of storage in blood stored in ACD + adenosine. As the storage period approached the 100th day some hemolysis was clearly visible, although less marked than that in blood stored in ACD alone, and further lysis of cells occurred when the cells were washed before transfusion. Correction for hemolysis was included in the calculation of viability when cells were stored for prolonged periods.

Inasmuch as blood stored in ACD + adenosine is bright red after several weeks of storage, in contrast to the darker color of blood preserved in ACD alone, the oxygen content and capacity [4] of such blood were measured. The results, recorded in Table IV, suggest a decreased oxygen utilization of red cells during storage in blood supplemented with adenosine. Concomitant analyses of aerobic utilization of glucose also showed the improved metabolic activity of erythrocytes stored several weeks in ACD + adenosine.

TABLE IV

Oxygen content and capacity of human blood during storage *

Days storage	O_2 Content (*vol. %*)	O_2 Capacity (*vol. %*)	% Sat. with O_2	O_2 uptake† *μliters/hr./ml. RBC*
1 day:				
Control	10.42	14.67	71.0	116
Adenosine	12.19	14.58	83.6	117
3 weeks:				
Control	6.50	13.02	49.9	72
Adenosine	8.89	13.40	63.3	106
7 weeks:				
Control	3.74	12.20	30.7	46
Adenosine	6.50	12.37	52.6	103

* Blood was collected in ACD and divided into two aliquots: 1) Control; 2) 2,500 μmoles adenosine per 100 ml. RBC added after 1-day sampling and after 3 weeks' storage. Both aliquots were equilibrated initially with nitrogen, and sampling was accomplished under anaerobic conditions.

† Aerobic utilization of glucose in cell suspension system: Main compartment: 0.01 ml. of 1 per cent methylene blue, 0.30 ml. of 1.8 per cent glucose, 1.00 ml. of red cell suspension, 1.70 ml. of M/10 phosphate buffer, pH 7.5; center well: 0.20 ml. of 5N NaOH. O_2 gas phase: conventional Warburg apparatus; 37°. (Huennekens, F. M., Liu, L., Myers, H., and Gabrio, B. W., Unpublished observations.)

[4] We are indebted to Dr. Robert Bruce for these analyses.

TABLE V

Effect of glucose on the survival of blood stored with adenosine *

Days storage	Glucose *μmoles/100 ml. blood*	Survival of RBC (%)
1		
Cit.	490‡	
Cit.+G†	1,500	
Cit.+A†	490	
Cit.+G+A	1,600	
14		
Cit.	24	18
Cit.+G	800	70
Cit.+A	12	36
Cit.+G+A	1,160	82

* Human blood was collected in 4 per cent citrate (1 vol. +9 vol. blood) and divided into four aliquots: 1) Control citrated blood; 2) Added 1,010 μmoles glucose per 100 ml. blood; 3) Added 1,100 μmoles adenosine per 100 ml. blood; 4) Added 1,010 μmoles glucose and 1,100 μmoles adenosine per 100 ml. blood. All samples were stored for 2 weeks at 4°C.

† G = glucose; A = adenosine.

‡ 490 μmoles glucose per 100 ml. blood represents the amount of glucose endogenous in the whole blood in each sample.

II. *Adenosine as a substitute for glucose in blood preservation*

It has been shown (1) that adenosine can reverse the biochemical lesion of stored red cells in the absence of glucose. These results were obtained upon incubation of the aged cells with the riboside at 37°C. for one hour. In addition, Prankerd and Altman (8) have reported that erythrocytes can utilize adenosine in place of glucose to promote phosphate exchange when incubated over a six-hour period. It was of interest, therefore, to ascertain whether adenosine could replace glucose in maintaining viability of the red cell over a long period of storage at 4°C. The data shown in Table V indicate that, with only endogenous levels of glucose present in blood, there was almost complete glucose utilization in the presence of 1100 μmoles of adenosine per 100 ml. whole blood, and the preservation of citrated blood was improved only slightly. At higher levels of glucose supplementation, a sparing action of adenosine on the utilization of glucose and improved viability of red cells could be demonstrated.

Inasmuch as the level of adenosine employed in the previous experiment was about equimolar to glucose, it was thought that a replacement mechanism might require greater amounts of the riboside. Consequently, citrated rabbit blood was stored with excess adenosine for four weeks (total of 15,000 μmoles per 100 ml. RBC added at intervals). There was an appreciable sparing of glucose utilization in the presence of a large amount of adenosine. The viability of cells stored in citrate + excess adenosine was 59 per cent and that of citrate + glucose was 50 per cent at four weeks of storage. However, the combination of both glucose and adenosine resulted in even better survival (85 per cent).

III. *Effects of related nucleosides*

Nucleosides related to adenosine, such as inosine, deoxyadenosine, guanosine, and xanthosine, similarly stimulated a resynthesis of organic phosphates in the stored erythrocyte after one hour incubation at 37°C. (3). The implications of this and the mechanism of action of nucleosides will be discussed in a subsequent communication.

Inasmuch as significant reversal of the chemical changes of the storage lesion can be accomplished with purine nucleosides other than adenosine, and since adenosine has been found to have potentially toxic properties, the viability of red cells stored with other nucleosides was determined.

A detailed study of deoxyadenosine, the least effective of the nucleosides on the basis of *in vitro* studies, is shown in Tables VI and VII. When this substance was added at the beginning of storage only, the optimal level for viability appeared to be 2600 μmoles per 100 ml. RBC, regardless of the fact that the level of organic phosphate esters was maintained better in the presence of an excess of the compound. The effect

TABLE VI

Concentration of deoxyadenosine in the storage of human blood *

Deoxyadenosine added *(μmoles/100 ml. RBC)*	48 days' storage	
	Organic P *(μmoles/100 ml. RBC)*	Survival of RBC (%)
0	154	5
230	223	8
600	240	15
1,200	418	32
2,600	660	51
5,200	925	52

* Human blood was collected in ACD, divided into aliquots, supplemented with increasing concentrations of deoxyadenosine, and stored at 4°C. for 48 days.

TABLE VII

Storage of human blood in ACD+deoxyadenosine

	μmoles Nucleoside/100 ml. RBC		Nucleoabsorption (%)	Survival of RBC (%)
	Added initially	Other additions		
Exp. I. 42 da. storage				
Control				12
D*1	3,500		75	69
D2	3,500	7,000	69	74
Exp. II. 48 da. storage				
Control				12
A*	2,000		74	45
D1	2,000		73	41
D2	2,000	5,000	62	64

* D = deoxyadenosine; A = adenosine.

of the deoxyriboside was comparable to that of adenosine in prolonging viability of stored red cells. Similarly, it was most efficacious when added at intervals throughout storage in order to maintain a higher concentration of the nucleoside and a greater absolute absorption.

Blood stored with xanthosine resulted in a sediment formation and was not tested for survival.

IV. *Toxicity of nucleosides*

Preliminary studies were undertaken in order to evaluate the relative toxicity for animals of adenosine and its analogs. Of the effects reported, hypotension appeared to be the most important and sensitive for assay purposes (9). A study was conducted on the effects of the intravenous administration of commercially available nucleosides [5] as well as plasma and washed red cells from blood stored in ACD + adenosine.

The average minimal intravenous dose of adenosine producing a depressor response was 1 μmole in the rabbit and 10 μmoles in the dog. The average blood pressure fall was 80 mm. Hg in four to ten seconds after injection. The duration of the effect was variable, increasing with greater doses to as much as 200 seconds when 500 μmoles was given intravenously to a dog. There were no significant electrocardiographic changes in rabbits given 10 times the minimal hypotensive dose. The administration of amounts up to 100 times the minimal dose did not produce fatalities. The results were comparable in the two species.

[5] Obtained from Schwarz Laboratories, Mt. Vernon, New York.

Solutions of guanosine, xanthosine, and inosine did not cause an observable effect in amounts up to ten times the minimal hypotensive dosage of adenosine. A significant fall in blood pressure was observed in the dog after 400 μmoles of deoxyadenosine was given. Thus the relative hypotensive effect of deoxyadenosine was estimated to be less than 5 per cent that of adenosine. Adenosine-5-phosphate was equal to adenosine in toxicity but ribose and adenine were without effect. It was demonstrated further that injected plasma from blood stored 14 days in ACD plus adenosine produced transient and mild hypotension, while injection of washed intact erythrocytes was without effect.

DISCUSSION

The incubation of stored blood with adenosine and other nucleosides results in a repletion of cellular organic phosphates and an increased ability of the cells to utilize glucose aerobically (1). This restoration of the energy potential has been associated, as well, with improvement in osmotic fragility, an increase in intracellular potassium and improvement in post-transfusion viability (2). From the data in the present communication, it is evident that the addition of adenosine (2500 μmoles per 100 ml. RBC) as a supplement to ACD during the storage of blood at 4°C. prolongs the period of useful storage to about 40 to 50 days. Supplementation at weekly intervals was shown to improve viability over an additional period of time.

The use of citrate alone as a preservative is associated with a rapid loss of cell viability, while the addition of glucose prolongs the useful storage period from 5 to 15 days, and an acid-citrate-dextrose medium extends this period to 21 days. Although adenosine added to citrated blood also improves viability, supplementation with both adenosine and glucose provides the best results. This implies that adenosine and glucose are complementary and neither completely substitutes for the other.

The hypotension produced in rabbits and dogs with small amounts of adenosine (1 to 10 μmoles) indicates the potentially hazardous effects of this substance. Only a small amount of adenosine-

treated blood has been transfused and only after repeated washing of the cells. Related purine nucleosides were found to be less "toxic," but only deoxyadenosine was studied in some detail.

It has been suggested (1, 3) that nucleosides provide the necessary substrate through phosphorolytic cleavage, and that the resultant ribose-1-phosphate participates through the aerobic shunt pathway in the energy-yielding reactions to maintain the cell. The existence of a nucleoside phosphorylase in this reaction is implicit in the work of Dische (10) and has been isolated in purified form from human erythrocytes (3). Inasmuch as inosine and guanosine are split readily by this phosphorylase and adenosine only at a very slow rate, it would appear that the action of adenosine could be explained by an initial conversion to inosine and subsequent cleavage to ribose-1-phosphate and hypoxanthine (3). This conversion can occur by means of an adenosine deaminase which has been found in the erythrocyte (11, 12). Thus this reaction sequence would implicate inosine as the actual effective agent in erythrocyte preservation, and further studies with this nucleoside will be reported subsequently.

SUMMARY

The addition of adenosine to rabbit or human blood stored at 4°C. in acid-citrate-dextrose improves the preservation of erythrocytes. The chemical characteristics of the red cell are improved, and the effective period of storage is approximately twice as long as with acid-citrate-dextrose alone. In high concentrations adenosine without glucose extends the period of viability of citrated blood and reduces the glucose consumption. However, both adenosine and glucose are necessary to provide optimal cell preservation. A similar effect is produced by related purine nucleosides, such as deoxyadenosine. The relative toxicity of these compounds when administered intravenously, and a possible mechanism of action of adenosine in blood preservation are discussed.

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A COMPARISON OF THE METABOLIC AND CIRCULATORY EFFECTS OF EPINEPHRINE, NOR-EPINEPHRINE AND INSULIN HYPOGLYCEMIA WITH OBSERVATIONS ON THE INFLUENCE OF AUTONOMIC BLOCKING AGENTS [1]

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Interest in adrenal medullary function has been revived in recent years by the demonstration that the adrenal medulla produces two hormones, epinephrine and nor-epinephrine. Nor-epinephrine, which is also produced by adrenergic nerves (1), differs in its chemical structure from epinephrine by the absence of an N-methyl group, and is the probable precursor of epinephrine. The observation by Goldenberg, Faber, Alston, and Chargaff (2) that adrenal medullary extract from cattle (natural epinephrine, U.S.P.) contained up to 18 per cent nor-epinephrine was confirmed by Tullar (3) and Auerbach and Angell (4). Subsequently, nor-epinephrine was found in varying proportions in the adrenal medullary extracts of several mammalian species, including man, and it has been shown that the majority of pheochromocytomas contain larger amounts of nor-epinephrine than of epinephrine (5).

Although the cardiovascular effects of these hormones in man have been well established (6), there are few studies concerning their comparative metabolic effects. It is highly probable that in many of the early studies of the circulatory and metabolic effects of epinephrine, mixtures which contained varying proportions of nor-epinephrine were used, since it was demonstrated in 1948 that adrenal medullary extract from cattle produced a rise in blood sugar in the rabbit comparable to that obtained with a mixture of 75 per cent epinephrine and 25 per cent nor-epinephrine, rather than that obtained with pure epinephrine (7).

The similarity of the responses which occur during insulin hypoglycemia to those produced by the administration of epinephrine, and the presence of increased amounts of epinephrine in the blood and urine during hypoglycemia (8, 9), have led to the general belief that increased adrenal medullary secretion during insulin hypoglycemia is in part responsible for the symptoms produced and for spontaneous recovery of the blood glucose level. This is further suggested by the observation in rats that a rise in blood lactic acid, similar to that which results from the administration of epinephrine, also occurs during insulin hypoglycemia. The lactic acidemia induced by hypoglycemia can be prevented by adrenal medullectomy (10).

This study was undertaken 1) to evaluate certain metabolic and circulatory effects of pure epinephrine and nor-epinephrine in man and to compare these effects with those induced by insulin hypoglycemia, and 2) to determine the effect of an autonomic blocking agent on certain circulatory and metabolic changes which occur during insulin hypoglycemia in order to throw light on the mechanisms involved in these changes.

MATERIAL AND METHODS

All subjects were free of metabolic or cardiovascular disease and fasted for at least 12 hours without physical activity or sedation. Throughout the study, blood pressure was measured at one-minute intervals by the auscultatory method. Pulse rate was measured every five minutes and electrocardiograms were obtained at frequent intervals. Lactic acid and glucose (as total reducing substance) were determined in duplicate in copper tungstate filtrates prepared from venous blood. Glucose was measured by the Nelson method (11) and lactic acid by a modification of the Barker-Summerson method (12).

Solutions of pure l-epinephrine and l-nor-epinephrine [3] were made from the powdered form and ascorbic acid

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[3] Supplied through the courtesy of Dr. M. L. Tainter, Winthrop-Stearns, Ind.

TABLE I

The effect of epinephrine on blood lactic acid and blood glucose

Subject	Epinephrine dosage *mcg./kg./min.*	Blood lactic acid *mg. per 100 cc.* Time after infusion started (*min.*) 0*	15	30	45	60	75	Blood glucose *mg. per 100 cc.* Time after infusion started (*min.*) 0*	15	30	45	60	75
P. S.	.085	7.8		17.4	23.7	30.2		75		129	159	154	
R. R.	.085	7.5		15.3	16.0	12.8		104		148	162	154	
R. J.	.085	11.2	11.8	15.0	15.7	15.4	15.1	92	106	113	120	117	99
S. W.	.085	10.5	14.8	20.2	24.1	24.8	21.2	101	110	119	123	120	109
D. V.	.085	8.6	13.8	13.5	15.6	16.0	12.6	105	124	142	147	145	129
I. C.	.085	20.3	25.3	25.5	27.8	29.4	25.9	99	123	129	141	137	132
W. J.	.085	8.5	9.9	12.4	14.5	13.6		101	112	122	132	124	
R. T.	.085	13.7	21.3	26.1	29.1	28.9	23.6	95	121	134	150	149	136
Mean rise at indicated time			4.0	7.2	9.8	10.4	6.8		17.2	33	45.3	41	23
P. S.	.170	11.0	18.2	20.3	24.0	30.2	20.5	91	138	151	181	178	159
R. R.	.170	8.8		25.3	28.9	18.7		102		142	178	178	
R. J.	.170	12.2	15.3	18.4	21.8	21.8	19.7	93	110	123	135	142	120
S. W.	.170	12.5	20.1	27.3	38.0	38.2	37.1	101	113	148	132	122	110
D. V.	.170	8.8	17.4	18.2	18.3	16.7	16.3	104	132	148	171	172	155
I. C.	.170	17.2	26.1	33.0	36.1	35.4	31.8	102	127	151	156	156	133
W. J.	.170	12.7	25.5	23.1	28.9	30.0		98	106	124	142	146	
R. T.	.170	11.2	24.9	30.2	36.1	36.5	21.7	107	141	152	172	172	155
Mean rise at indicated time			8.8	12.7	17.2	16.6	12.4		24.4	42.6	58.6	58.5	39.0
Mean difference in rise with the two doses			5.1	5.5	7.4	6.3	6.1		3.5	9.6	13.4	17.5	10.6
S.E.			1.36	1.22	1.90	1.94	2.37		1.82	3.54	2.09	3.42	5.57
p			<.02	<.01	<.01	<.02	<.1		<.2	<.05	<.001	<.01	<.2

* The values in 0 column are the mean of two determinations obtained during the control period.

was added as a preservative. Appropriate dilutions with saline were made from these stock solutions.

Following a one-hour control period, .085 mcg. per kg. per min. of epinephrine was administered intravenously with a Bowman constant infusion pump to eight subjects for a 50-minute period. Two blood samples were obtained during the control period and single samples at 15-minutes intervals during and after the infusion period. In order to evaluate dose responses, the infusions were repeated in the same subjects on a subsequent day under similar conditions except that the quantity of epinephrine was doubled (.170 mcg. per kg. per min.).[4]

The effects of nor-epinephrine .170 mcg. per kg. per min. were studied in a similar manner in four subjects. Double this dosage of nor-epinephrine was attempted, but had to be discontinued because of severe side effects.

In order to determine whether the circulatory and metabolic changes induced by administered epinephrine were affected by an autonomic blocking agent, hexamethonium[5] (100 mg.) was administered intravenously to three subjects before beginning an infusion of .085 mcg. per kg. per min. of epinephrine. Three additional subjects were given a rapid intravenous injection of 400 mg. of tetraethylammonium chloride[6] (TEAC) before and again during the infusion.

Insulin hypoglycemia was induced in five subjects with 15 units of regular insulin intravenously after a one-hour control period. Hypoglycemia was terminated with intravenous glucose 50 minutes later. Four blood samples were obtained during the control period, and single samples at ten-minute intervals during the insulin period, and at 15-minute intervals after glucose was begun. On a subsequent day, insulin hypoglycemia was again induced in the same subjects following the administration of 100 mg. of hexamethonium intravenously during the last ten minutes of the control period.

RESULTS

Striking differences in both the circulatory and metabolic effects of epinephrine and nor-epinephrine were observed. Epinephrine produced an increase in the pulse pressure accompanied by tachycardia and T-wave changes in the electrocardiogram. These circulatory changes were accompanied by an increase in blood glucose and lactic acid as summarized in Table I. Neither hexamethonium nor TEAC had any effect on either

[4] The doses of epinephrine and nor-epinephrine originally planned for this study were 0.10 mcg. per kg. per min. and 0.20 mcg. per kg. per min. Subsequent to completion of the study constant weighing and calculation errors were discovered which necessitated correction to the accurate dosage values.

[5] Hexameton chloride, supplied through the courtesy of Burroughs-Wellcome and Co.

[6] Etamon chloride supplied by Parke, Davis and Company, through the courtesy of Dr. E. C. Vonder Heide.

TABLE II

The effect of nor-epinephrine on blood lactic acid and blood glucose

Subject	Nor-epinephrine dosage *mcg./kg./min.*	Blood lactic acid Time after infusion started (*min.*) 0	15	30	45	60	75	Blood glucose Time after infusion started (*min.*) 0	15	30	45	60	75
J. H.	.170	7.9	9.6	9.4	9.2	10.4	10.2	77	99	84	82	77	75
M. M.	.170	16.1	16.1	11.1	12.0	10.8	10.6	109	131	135	141	121	105
B. S.	.170	21.3	14.7	10.2	9.8	9.3	9.3	103	119	127	122	111	99
J. B.	.170	8.3	8.6	8.4	8.5	8.5	8.2	115	120	127	124	120	113
Mean		13.4	12.3	9.8	9.9	9.8	9.6	101	117	118	117	107	98
Mean rise			−1.2	−3.6	−3.5	−3.6	−3.8		16	17	16	6	−3
S.E.			1.86	2.86	2.90	3.23	3.18		4.94	4.61	6.02	2.53	2.66
p			<.6	<.3	<.4	<.4	<.4		<.05	<.05	<.1	<.1	<.4

the circulatory or metabolic changes induced by infused epinephrine.

Nor-epinephrine produced a rise in both systolic and diastolic blood pressure, associated with bradycardia. There was a slight rise in blood glucose but no change in lactic acid (Table II). The effects of TEAC on the blood pressure changes induced by nor-epinephrine are presented in another report (13).

Circulatory and metabolic changes similar to those which occurred during the infusion of epinephrine were observed during insulin hypoglycemia. These changes were prevented by hexamethonium, as shown in Table III.

Epinephrine

The effect of epinephrine on blood pressure and pulse rate in eight subjects is shown in Figure 1. The infusion of .085 mcg. per kg. per min. resulted in a mean increase in systolic pressure of 10 mm. Hg and a mean fall in diastolic pressure of 13 mm. Hg; the mean pulse rate rose from 84 per minute to 97 per minute. Infusion of double this amount of epinephrine (.170 mcg. per kg. per min.) produced a mean increase of 19 mm. Hg in systolic pressure and a mean decrease of 12 mm. Hg in diastolic pressure, with a rise in pulse rate from 86 to 107 per minute. When the infusion

TABLE III

Effect of hexamethonium on response of blood lactic acid and blood glucose to insulin hypoglycemia

Subject	Blood lactic acid *mg. per 100 cc.* Time after insulin (*min.*) 0	10	20	30	40	50	65	80	Blood glucose *mg. per 100 cc.* Time after insulin (*min.*) 0	10	20	30	40	50
Insulin 15 units I.V.														
A. C.	14.8	12.6	15.8	17.7	25.3	29.9	27.1	24.9	123	105	71	53	47	62
J. S.	10.1	12.9	14.2	13.5	15.2	20.1	20.4	19.8	87	81	63	60	48	59
M. N.	15.2	13.8	17.1	16.4	16.6	20.7	19.0	17.0	97	79	47	35	33	39
R. J.	9.0	9.1	10.1	10.4	10.1	19.0	15.4	17.5	85	91	63	50	42	52
B. W.	8.4	10.5	9.2	10.0	11.7	13.7	13.2	15.2	87	93	73	47	34	48
Mean change at indicated time		+0.26	+1.77	+2.10	+4.29	+9.19	+7.51	+7.37		−6.0	−32.4	−46.8	−55.0	−43.8
Hexamethonium 100 mg. I.V. + insulin 15 units I.V.														
A. C.	13.2	12.6	13.7	16.8	18.3	18.0	19.9	23.8	98	75	38	20	22	21
J. S.	15.1	13.7	12.5	12.6	13.3	18.5	16.8	17.6	91	81	48	37	32	31
M. N.	13.6	13.5	14.2	18.8	15.9	14.5	15.7	16.4	95	92	55	34	23	22
R. J.	12.0	12.4	17.8	19.5	13.1	16.1	12.7	14.8	92	90	69	55	46	43
B. W.	9.5	10.0	9.4	10.2	10.0	6.7	9.0	11.1	121	114	84	62	49	41
Mean change at indicated time		−0.25	+0.87	+2.90	+1.44	+2.09	+2.13	+4.08		−9.0	−40.6	−57.8	−65	−67.8
Mean diff. in change with and without C6		+0.51	+0.90	−0.81	+2.84	+7.10	+5.38	+3.29		+3.0	+8.0	+11.0	+10.0	+24.0
S.E.		1.09	1.83	2.06	1.48	0.98	1.09	1.69		4.76	6.0	5.26	4.09	5.30
p		<.7	<.7	<.8	<.2	<.01	<.01	<.2		<.6	<.3	<.2	<.1	<.02

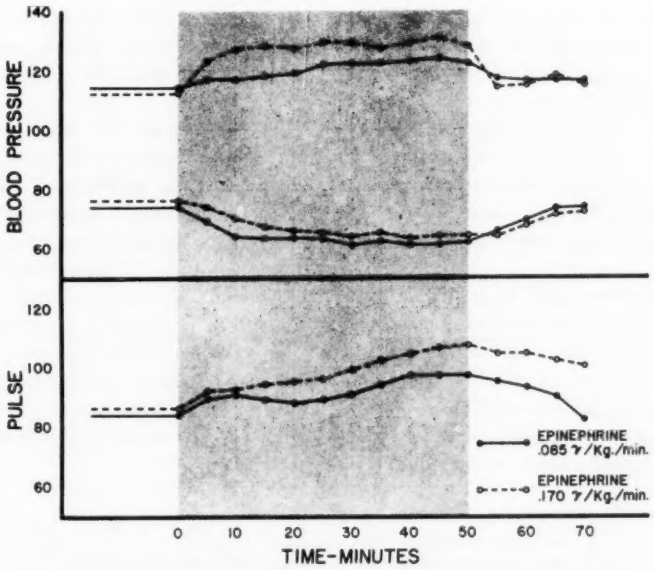

FIG. 1. THE MEAN BLOOD PRESSURE (MM. HG) AND PULSE RATE (PER MINUTE) RESPONSES TO EPINEPHRINE IN EIGHT SUBJECTS WHO RECEIVED .085 MCG. PER KG. PER MIN. ONE DAY AND .170 MCG. PER KG. PER MIN. ON A SUBSEQUENT DAY

The shaded area represents the infusion period.

was stopped, the blood pressure and pulse promptly returned to control values.

The electrocardiograms taken on six subjects during the infusion showed a decreased amplitude of the T-waves in four and inversion of the T-waves in two; the QT interval was prolonged in five. These changes were more marked with the larger dose, and disappeared in from thirty to sixty minutes after the infusion was discontinued.

The effects of epinephrine on blood glucose and lactic acid are shown in Figure 2. The peak rise in glucose occurred 45 minutes after the infusion was started; a mean rise of 45 mg. per cent was observed with .085 mcg. per kg. per min. and 59 mg. per cent with the larger dose. Comparisons of the rise in glucose above individual control values at the two-dose levels show a significant difference at 45 and 60 minutes, indicating a dose response relationship with respect to blood glucose.

The maximum rise in blood lactic acid occurred ten minutes after the infusion of .085 mcg. per kg. per min. of epinephrine was ended and five minutes before the infusion with the larger dose was ended. The smaller dose produced a mean increase of 9.8 mg. per cent above the control value, while the large dose resulted in a mean increase of 17.2 mg. per cent above the control value. The differences between the elevations of lactic acid obtained with the two doses of epinephrine are statistically significant at the 15, 30, 45 and 60-minute periods after the start of the infusion, and suggest a dose response relationship with respect to lactic acid.

Hexamethonium and TEAC had no effect on the circulatory or metabolic effects of epinephrine. In the three subjects who received hexamethonium prior to the infusion, blood glucose (average) increased 62 mg. per cent above the control level, and blood lactic acid 10.8 mg. per cent. Similar results were obtained in the three subjects who received TEAC before and during the infusion.

Nor-epinephrine

In Figure 3 are shown the mean changes in blood pressure and pulse rate in the four sub-

jects who received .170 mcg. per kg. per min. of nor-epinephrine. The mean rise in systolic pressure was 38 mm. Hg and 26 mm. Hg rise in diastolic pressure. The pulse rate decreased from 83 to 63 per minute. Changes in the T-waves and QT intervals seen with epinephrine did not occur. In one subject, nodal premature contractions appeared shortly after the infusion was begun but disappeared spontaneously after minutes of infusion.

Within a few minutes after beginning an infusion of .340 mcg. per kg. per min. of nor-epinephrine in these subjects, the mean blood pressure rose from a control level of 108/63 mm. Hg to 185/115 mm. Hg, an increase of 77 mm. systolic and 52 mm. diastolic. Simultaneously, severe throbbing headache and a sense of tightness in the chest developed, and the infusion was discontinued. The blood pressure rapidly returned to normal, although the headache persisted for as long as thirty minutes.

The effects of .170 mcg. per kg. per min. of nor-epinephrine on blood glucose and lactic acid are also shown in Figure 2 and Table II. The effects of the larger dose could not be evaluated because the infusion was discontinued after only a few minutes. A maximum mean increase in blood glucose of only 17 mg. per cent above the control value occurred 30 minutes after the infusion was begun; this rise was not significant and was only one-fourth that obtained with a similar dose of epinephrine. Nor-epinephrine had no effect on the blood lactic acid in any of the four subjects.

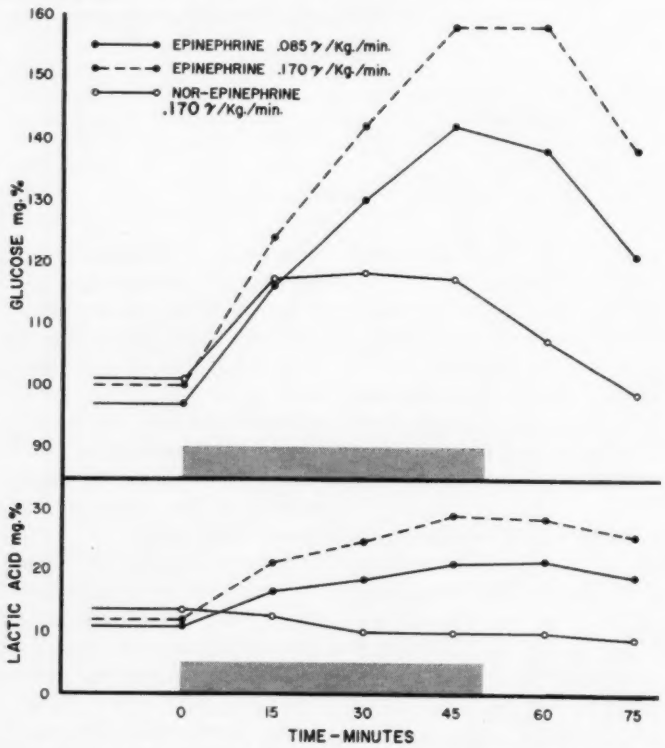

FIG. 2. MEAN VALUES OF THE RESPONSE OF THE BLOOD GLUCOSE AND LACTIC ACID DURING THE INFUSIONS OF EPINEPHRINE IN THE EIGHT SUBJECTS SHOWN IN FIGURE 1

In addition mean glucose and lactic acid responses during the infusion of nor-epinephrine are pictured. Again the shaded areas represent the infusion period.

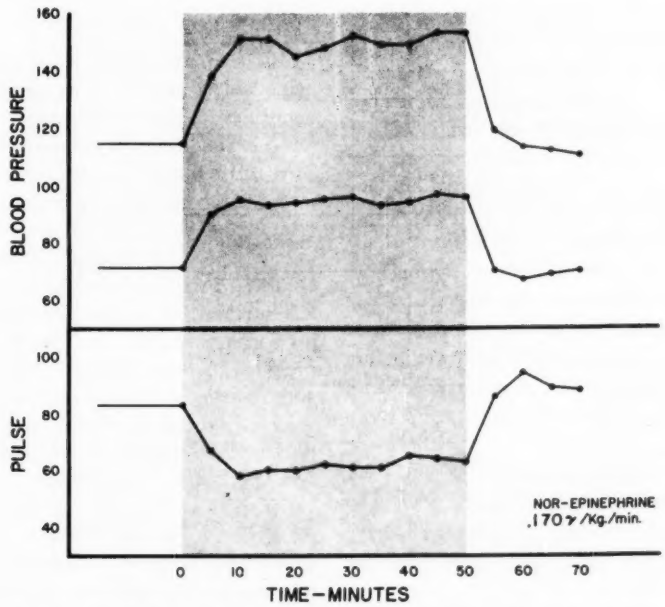

FIG. 3. MEAN BLOOD PRESSURE AND PULSE RATE RESPONSES IN FOUR SUBJECTS WHO RECEIVED .170 MCG. PER KG. PER MIN. OF NOR-EPINEPHRINE

The mean blood glucose and lactic acid responses in these subjects are depicted in Figure 2.

Insulin hypoglycemia

The circulatory changes which occurred during insulin hypoglycemia and the effect of hexamethonium on these changes are shown in Figure 4. Between 20 and 30 minutes after the administration of insulin, profuse sweating, hunger sensations and mild palpitation occurred in all subjects; simultaneous with these symptoms, the systolic blood pressure began to rise, the diastolic began to fall, and the heart rate increased. The T-wave in the electrocardiogram decreased in amplitude or inverted. The maximum changes in blood pressure and pulse were observed a few minutes before glucose was given, and pulse increased from 75 to 90 per minute. The circulatory changes and symptoms disappeared rapidly after the hypoglycemia was terminated; however, the T-wave changes persisted for as long as one hour.

During and following the intravenous administration of hexamethonium, all subjects developed signs of autonomic blockade as manifested by dryness of the mouth, dilatation and fixation of the pupils to light, absence of sweating, and increase in the pulse rate and a slight fall in blood pressure. Following the administration of insulin and during the hypoglycemic period the signs of autonomic blockade were maintained. The blood pressure, which continued to fall and reached its lowest level at the time when widening of the pulse pressure occurred with insulin alone, showed a mean maximum fall of 28 mm. Hg systolic and 20 mm. Hg diastolic from the control level. There was no further increase in pulse rate from that produced by the hexamethonium. Changes in the electrocardiogram similar to those observed with insulin alone also occurred. Hunger sensations were not abolished but were milder.

In Figure 5 are shown the metabolic changes which occurred during insulin hypoglycemia together with the effect of hexamethonium on these changes. Following insulin alone, the mean glucose level showed a steady fall during the first 40 minutes of the hypoglycemic period and then rose spontaneously before intravenous glucose was given. Blood lactic acid rose to 20.7 mg. per cent

(a significant rise from the control value) at the same time the spontaneous rise in glucose occurred.

After autonomic blockade with hexamethonium, insulin produced a greater hypoglycemic effect. During the last ten minutes of the insulin period, the blood glucose continued to fall, in marked contrast to the spontaneous rise observed at this time with insulin alone. Despite the further fall in glucose, the mean maximum rise in lactic acid during the hypoglycemic period was only 2.0 mg. per cent above the control value. As shown in Table III, individual variations in the lactic acid responses following blockade with hexamethonium showed considerable latitude in comparison with the changes in blood glucose, which were more consistent.

Changes in blood glucose similar to those observed in the present study with insulin alone and insulin with hexamethonium have been reported in man by Laurence and Stacey (14) and by Schachter in dogs (15).

DISCUSSION

In the first published report on the comparative hemodynamic effects of epinephrine and nor-epinephrine in man Goldenberg, Pines, Baldwin, Greene, and Roh (6) demonstrated that the pressor properties of these hormones result from different mechanisms. Epinephrine in doses sufficient to affect the blood pressure causes overall vasodilatation, a decrease in the peripheral resistance, a striking increase in the cardiac output and an increase in the heart rate due to direct cardiac stimulation. As a result, the systolic blood pressure becomes elevated but the diastolic pressure remains unchanged or falls. In contrast, nor-epinephrine causes overall vasoconstriction with consequent increase in the peripheral resistance, and no change in the cardiac output. Thus elevation of both systolic and diastolic pressure occurs. The heart rate is slowed by nor-epinephrine, presumably because of increased vagal tone, since the bradycardia may be abolished by atropine.

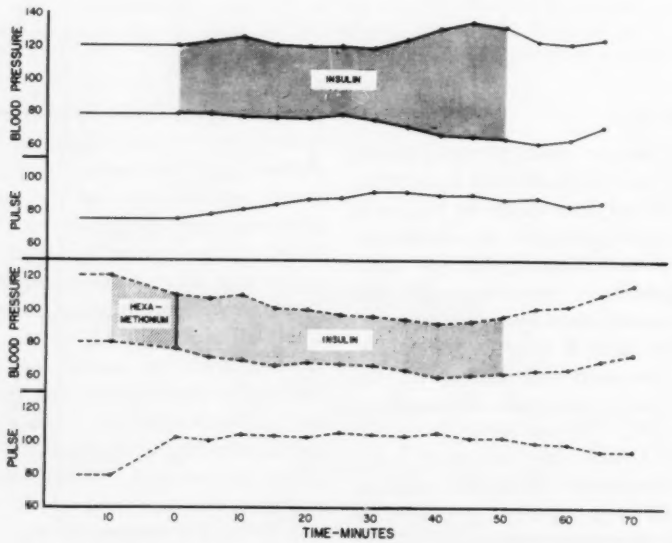

FIG. 4. THE MEAN BLOOD PRESSURE AND PULSE RATE CHANGES WHICH OCCURRED IN FIVE SUBJECTS DURING INSULIN HYPOGLYCEMIA ARE DEPICTED ON THE UPPER PORTION OF THE FIGURE. IN THE LOWER PORTION ARE THE CHANGES OBSERVED IN THESE SAME SUBJECTS WHEN THE SAME DOSE OF INSULIN (15 UNITS) WAS PRECEDED BY AUTONOMIC BLOCKADE WITH HEXAMETHONIUM (100 MG. INTRAVENOUSLY)

The shaded "insulin" areas indicate the period from injection of insulin to the termination of hypoglycemia by the administration of glucose.

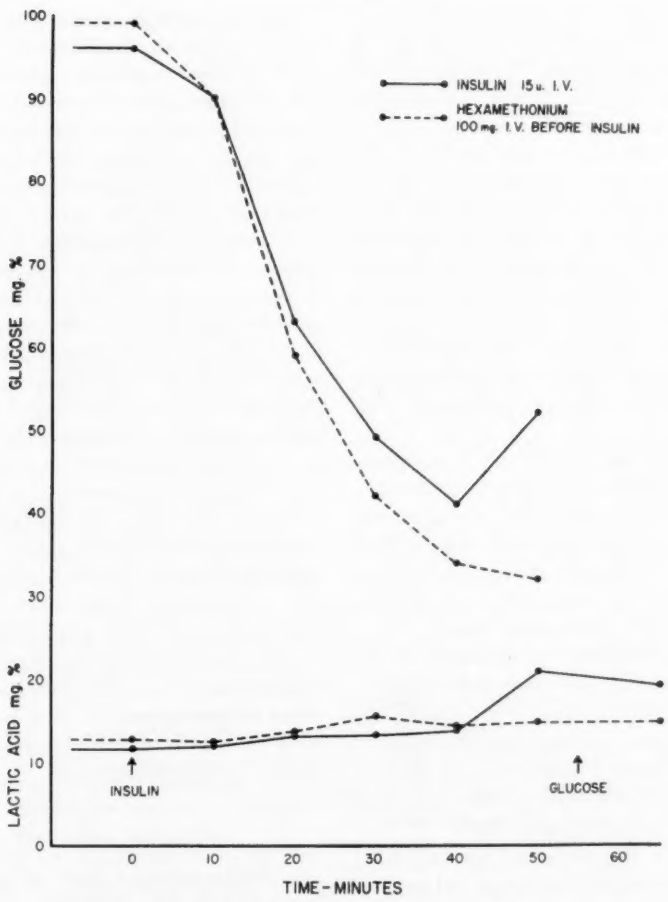

FIG. 5. THE EFFECT OF HEXAMETHONIUM ON THE BLOOD GLUCOSE AND LACTIC ACID RESPONSE TO INSULIN IN THE SAME SUBJECTS DEPICTED IN FIGURE 4

Note that after hexamethonium the hypoglycemic effect was greater, the blood glucose failed to rise at 50 minutes and the blood lactic acid rise was inhibited.

Electrocardiographic changes similar to those observed with epinephrine in this study have been reported by other investigators (16, 17) and may be related to changes in serum potassium or to the effects of this hormone on the myocardium (18, 19).

The circulatory and metabolic changes produced by infused epinephrine are quite similar to those observed here during insulin hypoglycemia, suggesting that adrenal medullary secretion of epinephrine is stimulated during hypoglycemia in man. Rise of the blood lactic acid and spontaneous recovery of the blood sugar curve are thereby accounted for on the basis of an induced epinephrinemia. The release of epinephrine in significant amounts from the adrenal medulla appears to be related to the degree of hypoglycemia. To judge from the blood lactic acid responses here presented, the secretion of epinephrine has begun when the blood glucose concentration reaches levels of approximately 50 mg. per cent.

In the rat, mild insulin hypoglycemia does not

affect the blood lactic acid; however, there is a very definite increase during moderate or severe hypoglycemia. In the adrenal demedullated rat, lactic acid does not increase during hypoglycemia and the degree of hypoglycemia produced by a given dose of insulin is greater than in the intact animal (10). Although the blood glucose level ultimately recovers, the recovery period is considerably prolonged in comparison with that of the intact animal. Quantitatively, the changes which occurred during insulin hypoglycemia in the subjects in the present study were approximately the same as those observed during the first fifteen minutes of the intravenous administration of .170 mcg. per kg. per min. of epinephrine (Figures 2 and 5).

Both hexamethonium and TEAC block transmission through autonomic ganglia. Hexamethonium was given before insulin because the blockade persists for well over one hour, whereas that produced by TEAC is much shorter in duration. The prevention by hexamethonium of the epinephrine-like changes induced by insulin hypoglycemia may be interpreted as indicating inhibition of adrenal medullary secretion of epinephrine, since neither hexamethonium nor TEAC affected the responses to administered epinephrine. The most likely site of block is the medulla itself, since it may be considered a modified ganglion and is supplied by pre-ganglionic fibers.

Billington, Paton, Reynolds, and Sherlock (20) in similar studies used intramuscular hexamethonium bromide (1 mg. per Kg.) and intravenous insulin (0.1 unit per kg.) given simultaneously. They were unable to prevent arterial lactic acidemia and did not observe either enhancement of hypoglycemia or failure of spontaneous recovery of the blood sugar. However, they did note inhibition of the expected rise in hepatic glucose output and splanchnic oxygen consumption and thereby postulated a partial block of epinephrine release by hexamethonium. It appears likely that the smaller dose of hexamethonium they employed or slower absorption from the intramuscular injection (whereas insulin was given intravenously at the same time) produced an incomplete block or allowed epinephrine to be released from the adrenal medulla before effective hexamethonium blockade was achieved.

The similarity of the electrocardiographic changes following insulin alone and insulin preceded by hexamethonium are of interest. The fact that these changes were not affected following hexamethonium despite the prevention of other changes during hypoglycemia suggests that epinephrine was not responsible and that some other mechanism may be involved.

The release of epinephrine by the adrenal medulla as an immediate or emergency response to moderate or severe hypoglycemia in man seems demonstrated by our data. The further fall in blood glucose and inhibition of the lactic acid response by hexamethonium indicate that this emergency response was prevented by blockade of the adrenal medulla. It is probable that ultimate recovery of the blood glucose level by other mechanisms would have occurred had the hypoglycemia not been terminated. It has been suggested that one such mechanism may be a direct glycogenolytic response of the liver to hypoglycemia (21); this may be the major factor which determines spontaneous recovery of the glucose level (22).

The observation that the blood lactic acid levels rise in response to epinephrine but are not influenced by nor-epinephrine may be of aid in evaluating the relative amount of epinephrine production by pheochromocytomas.

SUMMARY AND CONCLUSIONS

1. A comparative study of the effects of epinephrine and nor-epinephrine on the blood pressure, pulse rate, electrocardiogram, blood glucose and lactic acid is presented.

2. Epinephrine influences the circulation mainly through its cardiac effects, while nor-epinephrine acts mainly on the peripheral vessels.

3. Epinephrine induces a significant rise in blood glucose and lactic acid while nor-epinephrine does not affect these significantly.

4. The reported circulatory and metabolic changes induced by insulin hypoglycemia are qualitatively similar to these seen with the administration of epinephrine.

5. Whereas the circulatory and metabolic effects of administered epinephrine are not influenced by ganglionic blockade of the autonomic nervous system with hexamethonium or TEAC, these same effects are prevented by hexametho-

nium followed by insulin hypoglycemia, suggesting that the main pharmacologic action of these agents on the adrenal medulla is a blocking one.

6. The electrocardiographic effects induced by epinephrine, nor-epinephrine and insulin hypoglycemia are presented.

7. Blood lactic acid levels may be of aid in recognizing the presence of pheochromocytomas which produce mainly epinephrine.

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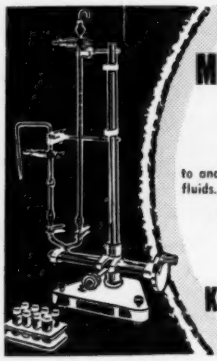

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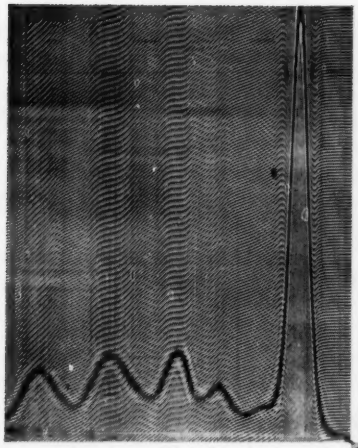

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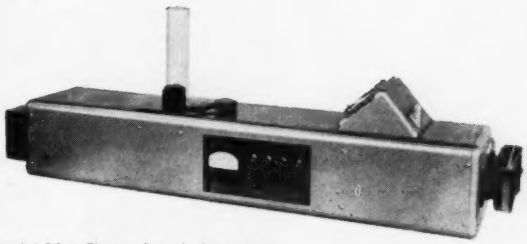

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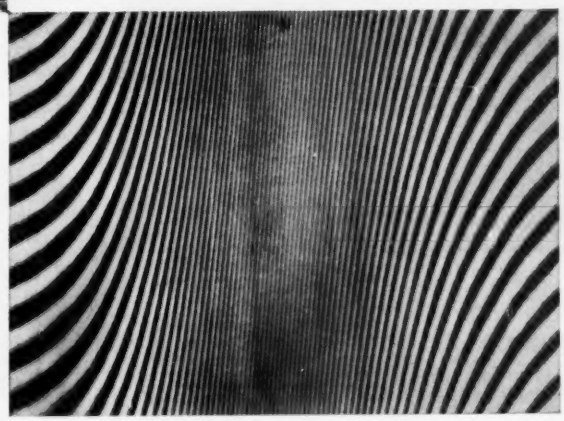

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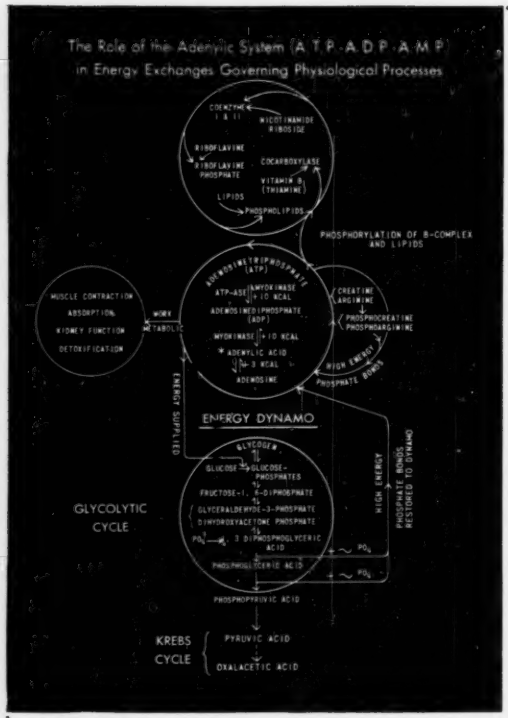

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The **NEW** Phenothiazine Derivative

Sparine*

For the Management of the Acutely Agitated Patient

• ***The acute alcoholic*** • ***The acute psychotic*** • ***The drug addict***

A promising new agent in chemopsychotherapeutics, **SPARINE** has demonstrated impressive effectiveness in controlling acute excitation without inducing significant side-reactions.[1,2,3]

SPARINE is a new, clinically effective phenothiazine derivative, which may be administered intravenously, intramuscularly, or orally. The route and dosage are determined by the extent of central-nervous-system excitation and by the patient's response.

Supplied: Tablets, 25, 50, and 100 mg., bottles of 50 and 500; 200 mg., bottles of 500. Injection, 50 mg. per cc., vials of 2 and 10 cc.

1. Seifter, J., et al.: To be published. 2. Fazekas, J.F., et al.: M. Ann. District of Columbia **25**:67 (Feb.) 1956. 3. Mitchell, E.H.: J.A.M.A. In press.

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